# DOCTOR OF PHILOSOPHY

# Beta-catenin Complexes in Colorectal Cancer

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# β-catenin Complexes in Colorectal Cancer

**Craig Donald Mackay**

Dissertation for the Degree of

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# Contents

## List of Figures

## List of Tables

## Declaration of authorship

I declare that I am the author of this work and that all cited references have been consulted. The content of this thesis is my own original work and any work carried out by others has been clearly stated in the text. This dissertation, in whole or in part, has not been previously accepted for a higher degree.

Signed _____ Date _____

Craig Donald Mackay

I certify that Craig D Mackay has spent the equivalent of at least nine terms in research work in the Division of Cell and Developmental Biology in the College of Life Sciences, University of Dundee, and he has fulfilled the conditions of the Ordinance General No. 39 of the University of Dundee and is qualified to submit the accompanying application for the degree of Doctor of Philosophy.

Signed _____ Date _____

Professor Inke Näthke

## Abstract

Genetic mutations that initiate and drive the progression of colorectal cancer cause defects in key signalling pathways that affect cell function. The Wnt signalling pathway is a key pathway that is disrupted in colorectal cancer and β-catenin is the key mediator of Wnt signalling. β-catenin is a multifunctional protein with roles in Wnt signalling and cell adhesion. I aimed to understand the relationship between the main interactions and functions of β-catenin. Specifically, I investigated how a stabilising mutation in β-catenin, decreases in APC, E-cadherin or PTEN altered the interactions of β-catenin and cell behaviour.

I established that mutant, non-degradable β-catenin can be sequestered by APC and is less available for binding to and stabilising E-cadherin than wild-type β-catenin, which only transiently interacts with APC. I found that, similar to the β-catenin/E-cadherin protein complex, the β-catenin/APC complex also localises to the plasma membrane and cultured cells and tissue organoids.

I also established a direct role for APC in directed cell migration that was independent of other genetic changes commonly found in colorectal cancer, including loss of PTEN, which on its own increased migration. To compare results from a cell culture to the situation in human tumours, localisation of β-catenin was measured in adenomatous and serrated human colorectal polyps. In all polyps, the predominant location of β-catenin was the plasma membrane, even in polyps with a serrated histology and reduced PTEN signals. However, the changes in E-cadherin observed in cultured cells lacking PTEN were not reproduced in these tumours. I also discovered that changes in transcription did not always mirror changes in protein expression, suggesting a much more complex relationship between the two main functions of β-catenin than expected.

Together, my data reveal that the distribution of β-catenin between its main functions as an adhesion or transcriptional regulator, cannot be predicted by the mutation status of APC or β-

catenin alone, but is affected by many other signalling pathways that are frequently dis-regulated in cancer.

## Acknowledgements

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## Abbreviations

| | |
|---|---|
| Ab | Antibody |
| ABR | APC binding region |
| Akt | Protein kinase B |
| APC | Adenomatous Polyposis Coli |
| Arm | Armadillo |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| α-cat | α-catenin |
| β-cat | β-catenin |
| CK1 | Casein kinase 1 |
| Co-IP | Co-Immunoprecipitation |
| Cy3 | small organic molecule excited at 550 nm |
| Cy5 | small organic molecule excited at 633 nm |
| DMSO | Dimethyl sulphoxide |
| Dsh/Dv1 | Dishevelled |
| DTT | Dithiothreitol |
| E-cad | E-cadherin |
| EDTA | Ethylenediaminetraacetic acid |
| EMT | Epithelial to Mesenchymal Transition |
| FACS | Fluorescence activated cell sorting |
| FAP | Familial adenomatous polyposis coli |
| FBS | Foetal Bovine Serum |
| FOBt | Faecal occult blood testing |
| Fz | Frizzled |

| | |
|---|---|
| GSK3β | Glycogen Synthase Kinase 3β |
| HNPCC | Hereditary non-polyposis coli |
| IF | Immunofluorescence |
| Ig | Immunoglobulin |
| IHC | Immunohistochemistry |
| IP | Immunoprecipitation |
| KD | Knockdown |
| K-Ras | Kirsten rat sarcoma viral oncogene homolog |
| LEF | Leukocyte enhancing factor |
| NES | Nuclear Export Sequence |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PenStrep | Penicillin/Streptomycin |
| PIP2 | phosphatidylinositol (4,5)-bisphosphate |
| PIP3 | phosphatidylinositol (3,4,5)-trisphosphate |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |
| SDS | Sodium dodecyl sulphate |
| TCF | T-cell factor |
| TP53 | Tumour protein 53 |
| Tris | Tris(hydroxymethyl) aminomethane |
| Vol | Volume |
| Wnt | Wingless int-1 |

# 1 Introduction

## 1.1 Colorectal Cancer

Colorectal cancer is the fourth most common cancer in the United Kingdom with approximately 41,600 diagnoses annually (CRUK 2015). In Scotland, around 3,500 patients are diagnosed with bowel cancer every year and it is the second most common cause of cancer death (COWG (2000)). Males in the United Kingdom have a 1 in 16 lifetime risk of a colorectal carcinoma diagnosis, while the risk in females is 1 in 20 (CRUK 2015). Bowel cancer risk increases with age and over 80% of diagnoses are in patients over the age of 60. A history of bowel cancer in close relatives is also associated with increased risk, as are lifestyle factors such as smoking, obesity and a western diet (CRUK 2015).

Current treatment has variable success. Radical surgical excision can offer a cure if the disease is at an early stage, but unfortunately does not in patients with advanced or metastatic disease. Surgery is combined with chemotherapy and/or radiotherapy. Most chemotherapeutic agents interfere with DNA or RNA synthesis, (Dihlmann and von Knebel Doeberitz 2005) preferentially destroying rapidly dividing tumour cells, but side effects are common due to off-target effects on normal cells. Despite recognition that significant clinical and tumour heterogeneity exists in colorectal cancer, current therapy remains largely non-specific. Individualised, targeted therapies based on features of both the tumour and patient may prove beneficial. Currently, this strategy is unavailable in the majority of cases.

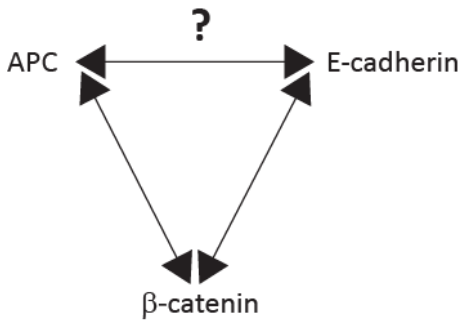
Survival outcome in colon cancer is heavily influenced by disease stage and an overall five year survival rate of 60% is achieved. For lesions diagnosed at an early stage this increases to over 95%, but for cancers diagnosed at an advanced stage, five year survival is less than 10%(CRUK 2015). Colorectal cancers are thought to develop from benign precursor polyp lesions that undergo malignant transformation over time (Fearon and Vogelstein 1990). This presents an

opportunity for detection at an early stage, which has prognostic benefits. The potential benefits of early detection prompted the implementation of population-based bowel screening programmes using guaiac-based faecal occult blood testing (FOBt). Early studies assessing FOBt-based bowel screening in the UK identified an increase in the proportion of early stage tumours (Hardcastle *et al.* 1996, Team 2003). The benefits of this screening modality have been confirmed in randomised controlled trials and a meta-analysis showed a 16% overall reduction in mortality for screened patients (Mandel *et al.* 1993, Kronborg *et al.* 1996, Towler *et al.* 1998, Hewitson *et al.* 2008, Mackay *et al.* 2014).

The detection rate of early stage polyp cancers has increased as a result of the screening programme. Many of these lesions are resected endoscopically and found to have a focus of malignancy on pathological assessment, but as no lymph nodes are present in the resection specimen, full pathological staging cannot take place. These lesions are a management conundrum because predicting which patients have lymph node involvement and would therefore benefit from formal surgical resection is difficult. Studies to date have identified the presence of vascular invasion, tumour grade, tumour budding, depth of invasion and positive resection margins to be associated with lymph node involvement (Ueno *et al.* 2004, Mitchell and Haboubi 2008, Gill *et al.* 2013). A National Institute of Clinical Excellence (NICE) guideline in 2011 attempted to identify which treatment was the best for patients that had undergone local excision of stage I colorectal cancer (including polyps) and subsequently found to have unfavourable pathological prognostic features. Based on current available evidence, no formal recommendation could be made (Poston *et al.* 2011). A large nationwide study is currently under way in an attempt to provide robust evidence that can guide therapeutic decision making.

**1.2 General aims of the Thesis Project**

Mutations of APC and consequent changes in β-catenin lie at the core of colorectal cancer. Dysfunction of the Wnt signalling pathway and cell adhesion are linked to the initiation and progression of colorectal cancer. β-Catenin is central to both of these functions and does so through interaction with either APC or E-cadherin.




*β-Catenin forms complexes with both APC and E-cadherin. Formation of these two distinct complexes is mutually exclusive. It is unclear how the amounts or functions of APC and E-cadherin influence each other.*

This thesis aims to measure the impact of mutations commonly implicated in colorectal cancer on the distribution of β-catenin between complexes containing APC and complexes containing E-cadherin and how this impacts on cell function. Specifically, the manipulation of β-catenin, APC and PTEN was used to gain a deeper understanding of how the relationships of β-catenin with its key binding partners (APC and E-cadherin) may change in cancer and what consequences this has for the function of the cell.

A greater understanding of the molecular mechanisms that drive colorectal cancer may provide opportunities to develop improved diagnostic and screening modalities or specific targeted therapies.

## 1.3 The gastrointestinal tract

### 1.3.1 Intestinal function

The main function of the intestine is the digestion of food and absorption of nutrients. A specialised epithelial surface comprises a barrier between the body and the intestinal luminal content (Tortora 1995). Within the gut epithelium, there are several resident specialised cell types with functions related to secretion, hormone production and self-renewal. The most common cell type is the absorptive enterocyte. These cells are primarily responsible for the uptake of nutrients from the bowel lumen and their transport to the capillaries in the submucosa. The cells are polarised with microvilli at their luminal surface to increase absorptive surface area and organised cell–cell junctions where adhesion complexes are located (Brittan and Wright 2004b). Alongside the enterocytes are mucous secreting goblet cells and enteroendocrine cells, which release hormonal signals to regulate intestinal function(Heath 2000) (Figure 1.1).

In both the small and large intestines, the epithelium is characterised by invaginations called crypts of Lieberkuhn. In the small bowel, finger-like projections called villi extend into the lumen, which increases the surface area for nutrient absorption. There are no villi in the large intestine and the crypt opening, also referred to as the 'collar,' lies flush with the intestinal lumen.

### 1.3.2 The colonic crypt

The colonic crypt unit contains numerous specialised epithelial cell types as well as self-renewing stem cells. In total, a crypt contains approximately 250 cells (Marshman *et al.* 2002). Two populations of stem cells are thought to reside in the crypt and can be identified by the marker leucine-rich G protein-coupled receptor 5 (Lgr5). A crypt base population as well as a slower cycling population of stem cells can be found in the +4 position in the crypt (Simons and Clevers 2011)Self-renewing stem cells are resident at the crypt base and give rise to all cell types resident

in the crypt (Leushacke and Barker 2012). Daughters of the stem cells undergo cell division in the transient amplifying zone prior to terminal differentiation into terminal specialised epithelial cells(Brittan and Wright 2004a). Differentiated cells migrate up the crypt and are then shed into the lumen after 3–4 days(van der Flier and Clevers 2009). The Paneth and stem cells remain resident at the crypt base (Figure 1.1).

Normal functioning of the crypt relies on the integrated co-ordination of proliferation, differentiation, migration, adhesion and exfoliation into the bowel lumen. Perturbations in any of these key cell functions can potentially lead to the initiation and subsequent progression of cancer.

Genetic mutation can trigger disruption of the normal crypt function and architecture leading to the formation of early-stage precursor lesions termed aberrant crypt foci. An accumulation of genetic defects in these lesions can drive progression of carcinoma through the development of colonic polyps and eventually carcinoma.

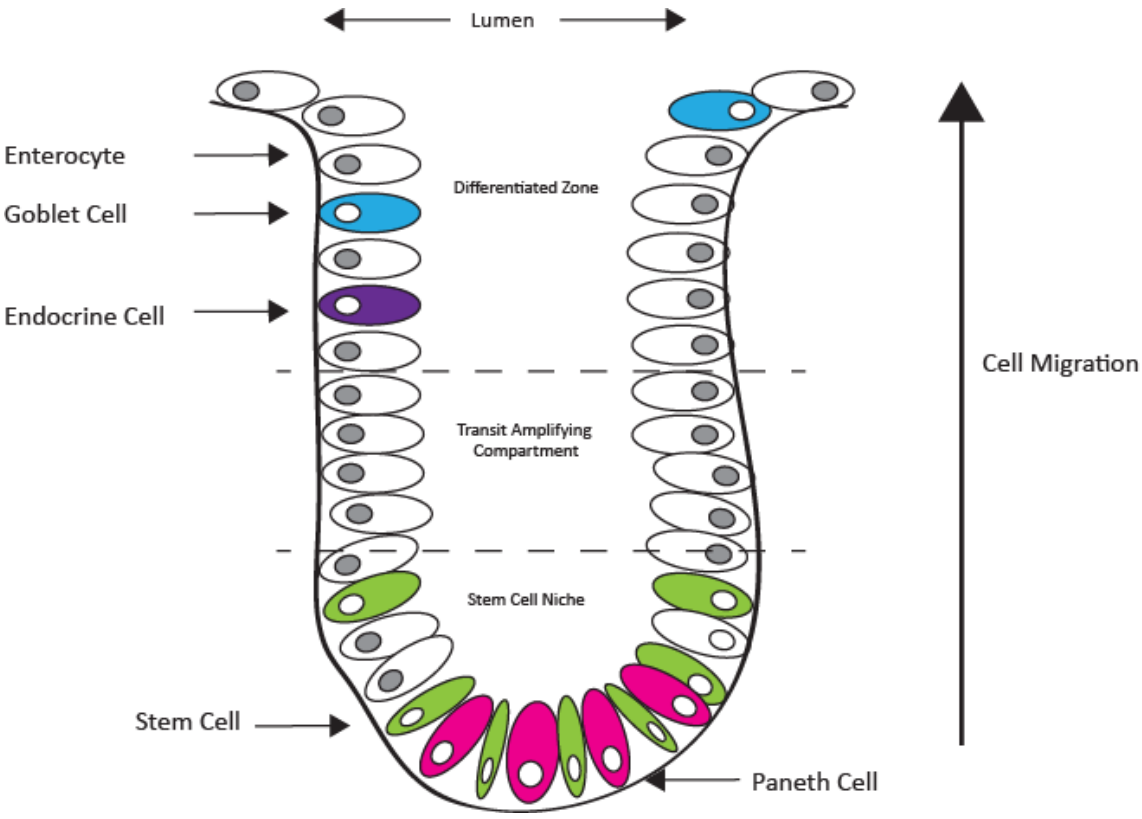



*Figure 1.1: Schematic depiction of an intestinal crypt. Stem cells (green) and Paneth cells (pink) are resident in the stem cell niche at the base of the crypt. Stem cell progeny undergo further cell divisions in the transit amplifying compartment prior to terminal differentiation into specialised epithelial cell types: enterocyte (white), goblet cell (blue) and endocrine cells (purple). Cells migrate up the crypt prior to being shed in the gut lumen.*

### 1.4.1 Pathogenesis of colorectal cancer

The transformation of normal colonic epithelium into cancer is thought to progress via accumulation of genetic mutations that cause changes in the histological appearance and structure of the tissue. The earliest microscopic changes, termed aberrant crypt foci, progress into benign polyps, defined as abnormal protrusions of the mucosa into the bowel lumen (Williams *et al.* 2013). Early polyps are benign, but progression results in malignancy when cells breach the muscularis mucosa (Lipper *et al.* 1983, Morson *et al.* 1984). Approximately 10% of polyps will progress to cancer (Hisabe *et al.* 2014) and an estimated 35% of patients will develop further polyps following removal within five years (Neugut *et al.* 1985, Winawer *et al.* 1993).

Robust indices that could predict which polyps are likely to progress and/or recur would be clinically beneficial in both management and surveillance strategies.

### 1.4.2 Classification of colorectal polyps

Colorectal polyps are classified according to three main categories. The first is based on histological features of the polyp, the second is related to specific mutations in the polyp and the third relates to mechanisms of conferring genetic instability into tumours.

The first classifier refers to the histological appearance of the polyp. In this thesis, reference is made to two main histological categories; conventional adenomatous polyps and serrated adenomas. Conventional adenomas are sometimes referred to as developing along the traditional or conventional pathway while serrated adenomas are described as being on the serrated or alternative pathway of carcinogenesis.

Conventional adenomas can be further subdivided by histological type into tubular, tubulo-villous or villous. End-stage carcinomas produced by this pathway are classified as adenocarcinomas. The second category is serrated adenomas, which are composed of sessile serrated adenomas, traditional serrated adenomas and hyperplastic polyps. The serrated adenoma pathway is sometimes referred to as the alternative pathway.

The second main category of classification is based on specific molecular characteristics that refer to mutations in particular genes such as APC, P53, BRAF, KRAS or PTEN.

The third category is based on more global molecular features of the lesion. This includes two mechanisms that confer genetic instability to a tumour. Chromosomal instability is the first of these and refers to aberrant rearrangements or duplications of chromosomes. The second is termed microsatellite instability. This is used to indicate genomic instability. This is often caused by mutation or methylation of DNA mismatch repair genes MLH1 or MSH2. This is sometimes referred to as the mutator phenotype. The last classification in this category is the CpG island

methylator genotype (CIMP). A CIMP-high tumour is characterised by extensive methylation, which can act to silence important tumour suppressor genes.

The two main histological categories of colorectal polyps, conventional adenomatous polyps and serrated polyps, are broadly characterised by distinct features. Conventional adenomas are characterised by the accumulation of mutations in key tumour suppressors and oncogenes. This promotes progression of benign lesions to cancer and is termed the adenoma–carcinoma sequence(Fearon and Vogelstein 1990). These lesions are characterised by early mutations in adenomatous polyposis coli (APC) and subsequent mutations in P53, KRAS and SMAD4(Dickinson *et al.* 2015). These conventional lesions are characterised by chromosomal instability and are CIMP low (Figure 1.2).

Serrated lesions are less common than conventional adenomas and debate exists as to their exact classification and characteristics. Approximately 10% of colorectal cancers are serrated carcinomas, derived from serrated polyps (Makinen *et al.* 2001). Precursor lesions in serrated carcinomas are serrated polyps and hyperplastic polyps (Goldstein 2006). Distinct histological subtypes exist in serrated adenomas with some lesions displaying serrated dysplasia while others are characterised by conventional adenomatous dysplasia (Tsai 2014). Common mutations associated with the serrated pathway are in the BRAF gene, present in an estimated 80% of serrated cancers (Lochhead *et al.* 2014). K-ras mutation has also been associated with distal serrated lesions (Yamane *et al.* 2014). These lesions are also characterised by microsatellite instability (Jass 2007) and tend to be CIMP high (Jass 2007).

Although common in conventional adenomas, APC mutation is an infrequent finding in studies so far in serrated lesions (Uchida *et al.* 1998, Dehari 2001, Sawyer *et al.* 2002, Fu *et al.* 2012) although methylation of APC may occur in some serrated lesions (Kim *et al.* 2011).

Murine studies have also linked phosphatase and tensin homologue deleted on chromosome ten (PTEN) with the development of serrated lesions and potential functional interaction of PTEN, KRAS and APC. Wild-type mice and mice with a predisposition to adenoma development (Apc $^{fl/+}$) had intestinal specific deletion of PTEN and activation of KRAS. The (Apc $^{fl/+}$) mice, predisposed to adenoma, had a significantly reduced lifespan compared with APC wild-type mice and lesions had evidence of invasive carcinoma but no metastasis. However, the APC wild-type mice with deletion of PTEN and activation of KRAS developed sessile serrated adenomas, hyperplastic polyps as well as metastasising serrated carcinomas, characteristic of the human pathway of serrated carcinogenesis (Davies *et al.* 2014). A study by the same group also implicates PTEN in the conventional pathway of carcinogenesis. Intestinal deletion of PTEN alone caused no change in intestinal architecture. However, PTEN deletion in APC heterozygous mice significantly reduced the lifespan and led to the development of invasive adenocarcinoma compared with only adenoma development in APC heterozygotes with PTEN (Marsh *et al.* 2008).

PTEN mutation has also been associated with the serrated pathway in human cancers. An analysis of 489 cancers found PTEN mutation in 5.8%. Interestingly, the tumours with PTEN mutation were significantly associated with proximal tumours, microsatellite instability, CIMP high and BRAF mutation. These are all features associated with the sessile serrated pathway (Day *et al.* 2013). A recent genome wide analysis of 276 samples to characterise mutations found in colorectal cancer identified PTEN mutation in 4% of non hyper-mutated cancers (Cancer Genome Atlas 2012).

A schematic of both the conventional and serrated pathways of colorectal carcinogenesis is shown in Figure 1.2.

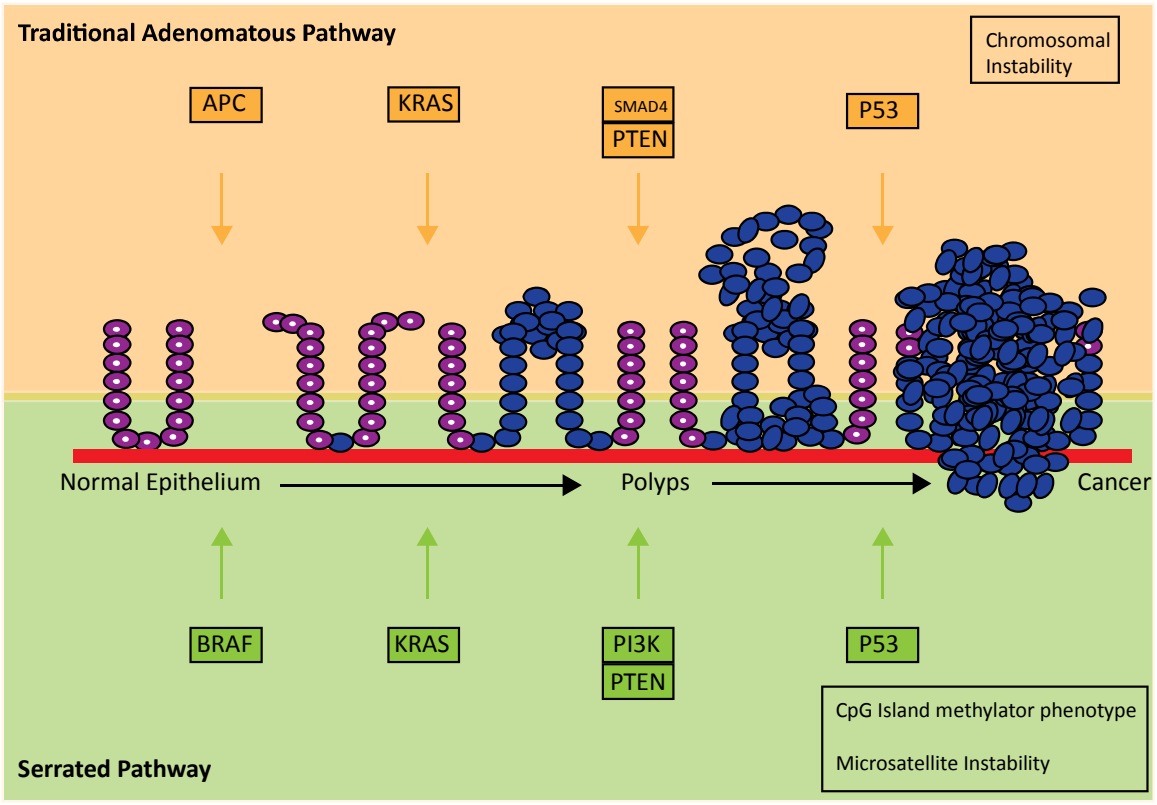



*Figure 1.2: A schematic of the adenoma to carcinoma sequence is shown. The top panel (orange) shows the conventional pathway and the genes commonly implicated in this progression along with chromosomal instability. The bottom panel (green) shows the serrated pathway and associated common mutations depicted with CpG Island methylator phenotype and microsatellite instability. This figure is a modification of (Dickinson et al. 2015).*

## 1.5 Adenomatous Polyposis Coli (APC)

### 1.5.1 The APC Protein

The *Apc* gene was identified on chromosome 5q21 as the genetic defect responsible for familial adenomatous polyposis coli (FAP). This autosomal dominant condition results in hundreds to thousands of benign colonic polyps, of which some inevitably undergo malignant change via the conventional pathway of colorectal carcinogenesis necessitating prophylactic colectomy in the majority of patients. Positional cloning of the FAP locus then resulted in identification of the *Apc* gene (Kinzler *et al.* 1991, Su *et al.* 1992).

2843 amino acids make up the large 312 kDa multifunctional APC protein. The N terminal domain contains two nuclear export sequences, binding sites for protein phosphatase 2a (PP2A) and microtubules (Nathke 2004).

The central portion of APC contains elements vital for regulating proteins in the Wnt signalling pathway. This region contains three or four 15 amino acid repeats and seven 20 amino acid repeats. These repeats bind β-catenin (Rubinfeld *et al.* 1996). Interspersed within these repeats are SAMP sequences that can bind to Axin (Fagotto *et al.* 1999). This mid-portion of APC is also a binding site for GSK3β. The overlapping binding sites for these important interactors are in the same region on APC, suggesting that binding may be mutually exclusive.

The C terminal end of APC contains two microtubule binding regions (Munemitsu *et al.* 1994, Smith *et al.* 1994, Dikovskaya *et al.* 2010) and is capable of binding to the microtubule end-binding protein EB1 (Askham *et al.* 2000) (Figure 1.3). This region also binds to actin.

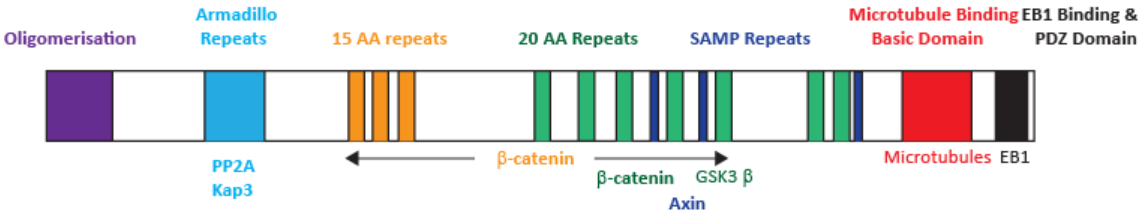



*Figure 1.3: A schematic representation of the APC protein. Binding partners are colour coded according to their recognised binding locations.*

### 1.5.2 The Functions of APC

Truncating mutations of APC are responsible for FAP and are also found in >90% of colorectal carcinomas (Fodde 2002). The high incidence of APC defects in colorectal cancers suggests that APC is vital in governing normal epithelial homeostasis. Mutations in this multifunctional protein may impact on many different cellular functions that can predispose to cancer if unregulated.

#### 1.5.2.1 APC in the canonical Wnt signalling pathway

APC is an essential component of the Wnt signalling pathway and modulates β-catenin (Figure 1.4). In the absence of a Wnt signal, β-catenin is targeted for degradation by the proteasome. This relies on the formation of a destruction complex composed of casein kinase 1 (CK1), GSK3β, protein phosphatase 2, the scaffold protein axin and APC. CK1 and GSK3β mediated phosphorylation of the scaffold protein axin, increases its binding affinity to β-catenin (Luo *et al.* 2007), increasing the efficacy of β-catenin phosphorylation by CK1 (Dajani *et al.* 2003). CK1 phosphorylates β-catenin at serine residue 45, priming it for subsequent sequential phosphorylation at serine residues 41, 37 and 33 by GSK3β (Yost *et al.* 1996, Liu *et al.* 2002). The phosphorylated β-catenin is targeted by β-TrCP E3 ubiquitin ligase and subsequently degraded by the proteasome (Hart *et al.* 1999).

Upon binding of a Wnt ligand to its receptor Frizzled, the destruction complex is inhibited. This prevents β-catenin degradation allowing its accumulation and translocation to the cell nucleus. Here, its interaction with T-cell factor (TCF) and Lymphoid enhancer factor (LEF) transcription

factors (Brantjes *et al.* 2002), stimulates transcription of a plethora of Wnt target genes (He *et al.* 1998, van de Wetering *et al.* 2002, Sansom *et al.* 2004) affecting the proliferation and differentiation of cells.

Truncation mutations in APC can result in the loss of β-catenin regulation, and this plays a key role in the initiation and progression of colorectal cancer.

#### 1.5.2.2 APC and Cell migration

APC also interacts with and stabilises microtubules via binding of its C-terminal region directly to microtubules or to the microtubule binding protein EB1 (Munemitsu *et al.* 1994, Nathke 2004). Microtubule functioning is important to the cytoskeleton, such as cell division and co-ordinated cell migration. APC mutation can negatively impact on these functions, contributing to cancer progression (Nelson and Nathke 2013) as co-ordinated, directional cell migration is required for normal function of the intestinal crypt. Normally, cells ascend the crypt and are shed into the intestinal lumen. Mutations causing migration defects can result in the cell having an increased residency in the crypt, allowing time for it to accumulate further mutations to drive cancer progression.

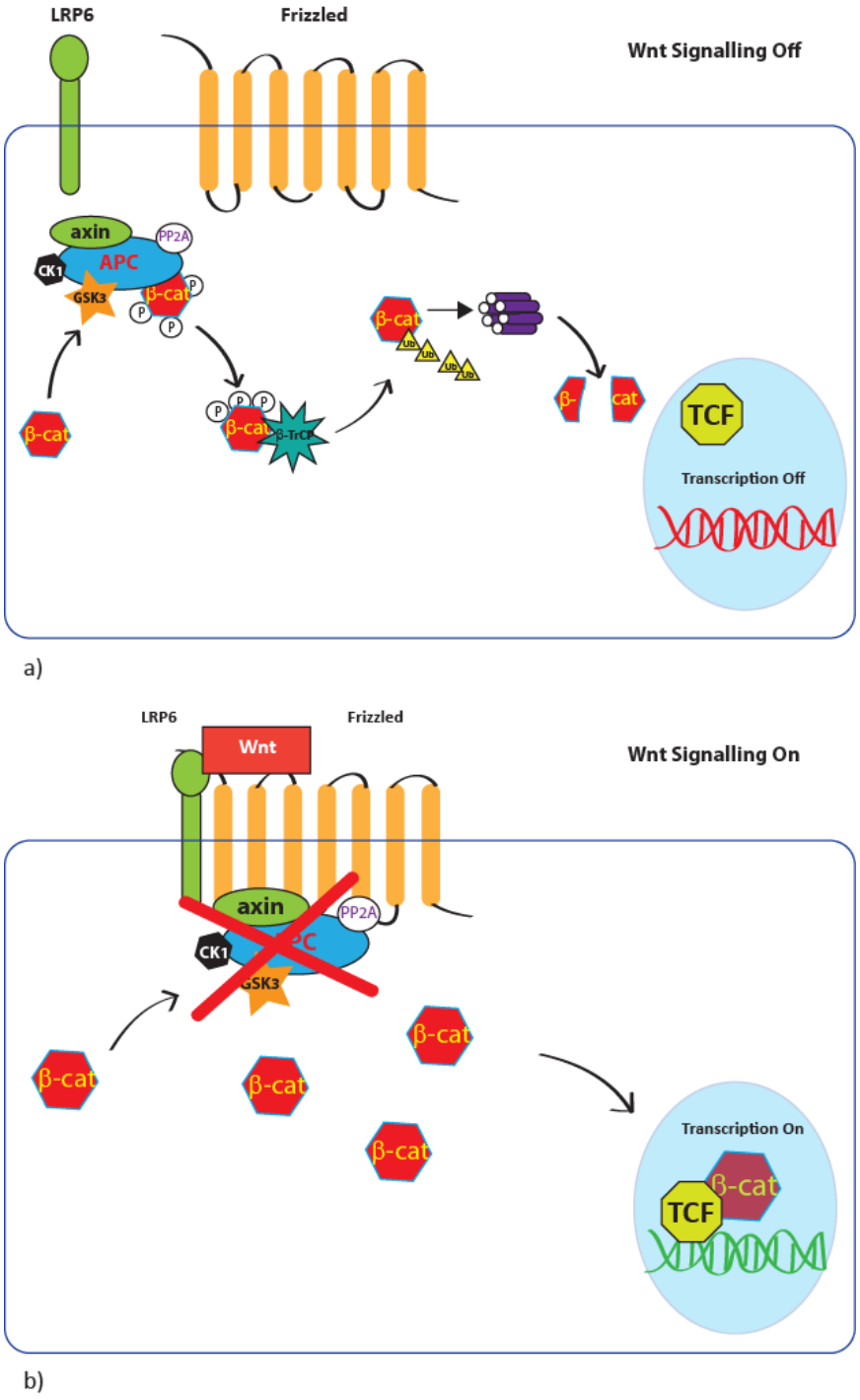



*Figure 1.4: Schematic representation of APC in the canonical Wnt pathway. In the absence of a Wnt signal (a) the destruction complex sequentially phosphorylates β-catenin, which targets it for β-TrCP-mediated ubiquitination and subsequent proteosomal degradation. Upon binding of a Wnt ligand (b) to LRP6 an interaction between the Wnt receptor and Frizzled causes inhibition of the destruction complex. This leads to accumulation of β-catenin and translocation to the nucleus where it activates transcription of Wnt target genes causing cell proliferation.*

## 1.6 β-Catenin

β-Catenin is a multifunctional protein with key roles in embryonic development as well as tissue homeostasis, renewal and regeneration in adult organisms (Valenta *et al.* 2012). It was discovered independently twice in relation to two of its most important functions, cell adhesion and Wnt signalling. It was initially isolated along with proteins associated with $Ca^{2+}$-dependent cell adhesion at adherens junctions, α-catenin, plakoglobin and E-cadherin (Ozawa *et al.* 1989). Studies of its *Drosophila* orthologue Armadillo revealed the signalling function of β-catenin. Armadillo mutants in *Drosophila* fail to develop the organised segments of denticles and belts in the Drosophila embryonic cuticle characteristic of the wild-type embryo (Wieschaus E 1984). Further studies found that the function of Armadillo in segmentation of the embryonic cuticle is regulated by wingless (Wnt) (Riggleman *et al.* 1990) thus identifying the role of β-catenin in the Wnt signalling pathway. The nematode *Caenorhabdidis elegans* has three distinct β-catenins. One has an adhesion specific role (HMP-2) and two are for signalling (BAR-1) & (WRM-1) (Liu *et al.* 2008). The ability of a single β-catenin to carry out two important but disparate functions in humans may partly relate to the protein structure.

### 1.6.1 Structure of β-catenin

β-Catenin is composed of 781 amino acid residues in humans. Twelve Armadillo repeats comprise the central region (Figure 1.5) with each repeat comprising 42 residues arranged in three helices. Together, the helices form a super helix that forms a positively charged groove providing a binding platform for APC, E-cadherin and TCF/Lef. This renders the binding of APC, E-cadherin and TCF/Lef mutually exclusive because their binding sites on β-catenin overlap (Valenta *et al.* 2012). The central region is flanked by N and C terminal domains, which may be structurally flexible (Huber *et al.* 1997). Transcriptional activity is performed by monomeric β-catenin whereas a β-catenin–α-catenin dimer associates with E-cadherin at adhesion junctions (Gottardi and Gumbiner 2004). E-Cadherin binding requires all 12 Armadillo repeats, whereas

interaction with TCF in the nucleus only requires the eight central repeats. Back folding of the flexible C-terminus could potentially impede binding to E-cadherin but not to TCF (Graham *et al.* 2000, Huber and Weis 2001). The C terminal domain has been shown to be essential for signalling but dispensable for adhesive function (Xing *et al.* 2008). Specific mutations in the Drosophila orthologue Armadillo disrupt adhesive function but do not impact on Wnt/Wingless signalling activity or vice versa (Orsulic and Peifer 1996). The location of β-catenin in the cell may also influence its function in either signalling or adhesion. Each of these functions will now be considered

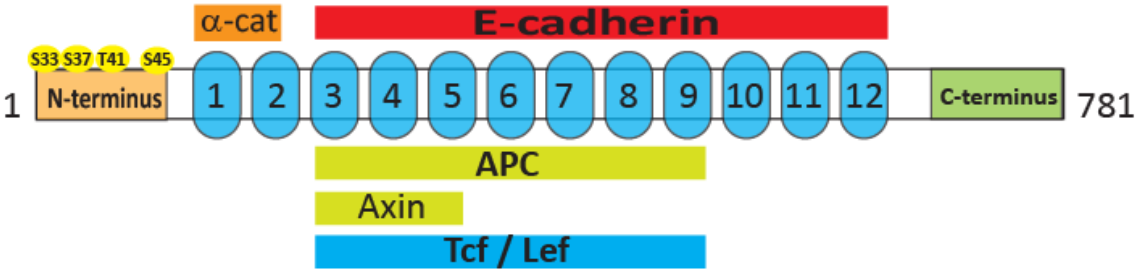



*Figure 1.5: Schematic representation of the β-catenin protein showing central Armadillo repeats flanked by the N and C termini. Important phosphorylation sites at S33, S37, T41 and S45 that mediate β-catenin destruction are also represented. Binding regions of E-cadherin, APC, Axin and TCF /Lef show overlap implying mutual exclusivity in binding.*

**1.6.2 Functions of β-Catenin**

1.6.2.1 β-Catenin in cell adhesion

β-Catenin was first identified in conjunction with E-cadherin at adhesion junctions. These junctions are critical in the formation of polarised epithelial tissues and the maintenance of tissue organisation (Meng and Takeichi 2009). Cadherins are transmembrane proteins that function in $Ca^{2+}$-dependent cell adhesion and are named according to the tissue in which they predominantly function. E-cadherin refers to epithelial cadherin, although other forms such as N-cadherin also exist (Meng and Takeichi 2009).

Interaction between E-cadherin and β-catenin is essential for effective formation of functional adherens junctions with β-catenin binding to the cytoplasmic tail of E-cadherin. The catenin–cadherin interaction is initiated while the newly synthesised cadherin is in the endoplasmic reticulum and the two proteins are transported as a unit to the plasma membrane (Hinck *et al.* 1994). Its association with E-cadherin also stabilises β-catenin, as this cadherin-bound pool cannot be targeted for destruction by the APC destruction complex (Huber and Weis 2001).

Cadherin-bound β-catenin can also bind α-catenin at distal segments of the N-terminus (Pokutta and Weis 2000). This simultaneous binding functionally links the adherens junction to the actin cytoskeleton via α-catenin. The β-catenin–α-catenin interaction forms a heterodimer, while as a homodimer α-catenin interacts with actin, promoting bundling of actin filaments (Benjamin *et al.* 2010). The association of β-catenin and α-catenin at adherens junctions therefore can impact on the dynamics of the actin cytoskeleton (Yamada *et al.* 2005).

#### 1.6.2.2 β-Catenin and the Wnt Signalling Pathway

β-Catenin is the key effector molecule of the Wnt signalling pathway. In the absence of a Wnt ligand, β-catenin is targeted for destruction by a multiprotein complex containing APC, axin, GSK3β and CK1 (Figure 1.4). Binding of a Wnt ligand to its receptor leads to inhibition of the destruction complex, resulting in stabilisation and accumulation of β-catenin. The β-catenin can then translocate to the nucleus and bind to TCF/Lef transcription factors. The binding of β-catenin converts TCF/Lef from transcriptional repressors to transcriptional activators, stimulating transcription of specific Wnt target genes (Najdi *et al.* 2011, Archbold *et al.* 2012).

##### 1.6.2.2.1 Wnt signalling in intestinal carcinogenesis

β-Catenin is central in maintaining normal intestinal tissue homeostasis and ordered structure of the colonic crypt (Figure 1.1). β-Catenin/TCF transduction of the Wnt signal is important in maintenance of the progenitor phenotype of crypt-base stem cells (Reya and Clevers 2005). Paneth cells are a source of Wnt (Farin *et al.* 2012) and establish a focused Wnt gradient in relation to the neighbouring stem cell (Sato 2013). The resultant high Wnt level in the stem-cell niche supports the self-renewing properties of the stem cells. Cells further up the crypt are exposed to a decreasing Wnt gradient, allowing escape from the self –renewing stem cell phenotype and differentiation into specialised epithelial-cell-type lineages (Sato 2013). In TCF4-null mice the crypt progenitor compartment is lacking, implying that its existence is dependent on Wnt signalling and is responsible for maintenance of self-renewing stem cells at the crypt base (Korinek *et al.* 1998). This is supported by studies that inhibit the Wnt receptor in adult mice using Dickkopf-1, which results in loss of crypts (Pinto *et al.* 2003, Kuhnert *et al.* 2004).

Dysfunction of β-catenin in the gut can have dire consequences and is tightly associated with carcinoma. β-Catenin is usually implicated in carcinogenesis following loss of APC, occurring in >90% of colorectal cancers. Some tumours lacking APC mutation have been found to have

activating mutations of β-catenin (Miyaki *et al.* 1999) (Johnson *et al.* 2005) and some authors suggest that APC and β-catenin mutation in tumours is mutually exclusive (Morin *et al.* 1997) (Sparks *et al.* 1998). β-Catenin mutations are far less common than loss of APC and are characterised by point mutation of N-terminal residues or deletion of exon 3 (Ilyas *et al.* 1997, Morin *et al.* 1997, Johnson *et al.* 2005, Segditsas and Tomlinson 2006). Like APC loss, these mutations result in the loss of important phosphorylation sites (residues 33, 37, 41 & 45) that are vital for the targeted degradation of β-catenin and one may intuitively expect the functional consequences to be comparable.

β-Catenin mutations are particularly associated with HNPCC, an inherited autosomal dominant condition characterised with DNA mismatch repair defects and a predisposition to cancer. One study identified β-catenin mutations in 43% of HNPCC tumours. Interestingly, none of the β-catenin mutant tumours had APC mutation (Miyaki *et al.* 1999), while a separate analysis found 18% of studied HNPCC tumours to have a β-catenin mutation.

In contrast, the mutation rate in sporadic cancers is less. No β-catenin mutations were identified in a series of sporadic cancers, although 1.2% of studied adenomas carried β-catenin mutations in the same series ((Johnson *et al.* 2005). Similarly, 12.5% of small adenomas were found to have a β-catenin mutation versus only 2.4% of large adenomas and 1.4% of sporadic cancers in a distinct series (Samowitz *et al.* 1999). This suggests that lesions harbouring mutations in β-catenin may be less likely to progress than those with loss of APC. Considering that the functional consequences ought to relate to the presence of stabilised β-catenin in both cases, the reasons for this are unclear. Nonetheless, these findings suggest tumours with either mutation of β-catenin or APC may progress along divergent paths with distinct outcomes.

Although uncommon in colorectal cancer, mutation in β-catenin exon 3 is found with more frequency in other tumour types such as hepatocellular carcinoma and pseudo papillary tumours of the pancreas (Huels 2015). In colorectal cancer mutations out with exon 3 at the C-

terminus of β-catenin can also be identified (Cancer Genome Atlas 2012). These mutations do not affect any of the key residues relevant for β-catenin stabilisation. It is unclear therefore if these are passenger mutations or if they can influence phenotype.

Loss of APC or activating mutations in β-catenin both lead to stabilised β-catenin. However the frequencies of these mutations in colorectal cancer are markedly different. The reasons for this are unclear but it may relate to the levels of Wnt signalling required to initiate tumour genesis in the gut. The pattern of APC mutations appear to be selected to provide an optimal but not maximal level of β-catenin transcriptional activation. This leads to favourable conditions to stimulate transformation (Fodde and Brabletz 2007, Buchert et al. 2010). Other authors have also suggested that in the presence of wild type APC an activating mutation may not be sufficient to drive carcinogenesis as the β-catenin can be bound and sequestered by E-cadherin (Huels 2015).

### 1.6.3 Crosstalk between Adhesion and Transcriptional regulation functions

β-Catenin has distinct roles in cell adhesion and transcriptional regulation. Interaction with E-cadherin or APC regulates this functional balance but their binding to β-catenin is mutually exclusive. How the cell determines how β-catenin is partitioned between adhesive and transcriptional functions is unclear, but crosstalk between the two is necessary (Figure 1.6). Studies in the 1990s showed that adhesion and Wnt signalling draw on the same pool of β-catenin and a bias towards one function can impact on the other.

Dorsal axis formation in the xenopus embryo is dependent on Wnt signalling, and this can be inhibited by overexpression of cadherins (Heasman *et al.* 1994). The wingless phenotype in drosophila, characterised by segment polarity defects, can be mimicked by overexpression of cadherin. This phenotype is due to an absence of wingless, which in turn leads to reduced levels of Armadillo (β-catenin), negatively impacting on its signalling function. These findings imply that E-cadherin can stabilise a pool of Armadillo (β-catenin) reducing its signalling role (Sanson *et al.* 1996). Armadillo (β-catenin) mutant drosophila have a less severe segment polarity defect if one E-cadherin allele is expressed compared with flies with two (Cox *et al.* 1996). The authors of this study proposed that expression of only one E-cadherin allele could free up wild-type β-catenin, allowing it to play more of a signalling role, thus reducing phenotypic severity. In SW480 colorectal cancer cells, cadherin expression triggered a translocation of β-catenin from the nucleus to the plasma membrane with an associated reduction in LEF-mediated transcription (Sadot *et al.* 1998). In a similar fashion, embryonic stem cells lacking E-cadherin display an accumulation of nuclear β-catenin and increased transcription of Wnt target genes. This phenotype was rescued by expression of wild-type E-cadherin but not E-cadherin lacking the β-catenin binding domain (Orsulic *et al.* 1999).

The influence of E-cadherin on β-catenin-mediated transcription may be influenced by activity of the Wnt pathway. DLD-1 colorectal cancer cells contain a truncating mutation in APC causing

activation of the Wnt pathway. siRNA-mediated depletion of E-cadherin in these cells results in dissociation of adherens junctions, translocation of β-catenin to the nucleus and augmentation of Wnt-mediated transcription. In contrast, the same treatment caused no effect on transcription in HaCaT keratinocytes, which lack Wnt activity. Forced expression of E-cadherin in fibroblasts that also lack Wnt activity alters cell morphology but does not impact on gene transcription (Kuphal and Behrens 2006). Thus, stabilisation of β-catenin by binding to E-cadherin at cell junctions can provide a reservoir for free cytoplasmic β-catenin to bind. Dissociation of the adherens junctions on the other hand can potentially release β-catenin from its association with E-cadherin, increasing the signalling function of β-catenin (Heuberger and Birchmeier 2010).

The participation of β-catenin in either a signalling or adhesion role may be influenced by specific phosphorylation signatures. Regulation by APC is reliant on the presence of several N-terminal residues that require phosphorylation for degradation to proceed. Likewise, stability of the adherens junctions is influenced by specific phosphorylation signatures. These residues are generally located towards the C-terminus of β-catenin, the opposite end of the molecule to those phosphorylation sites important to the function of APC. Phosphorylation of residues serine 686 and 692 increases the stability of the interaction between β-catenin and E-cadherin (Huber and Weis 2001). Alternatively, phosphorylation of residue tyrosine 654 decreases the affinity of β-catenin for E-cadherin, which may enhance signalling activity (Piedra *et al.* 2001).

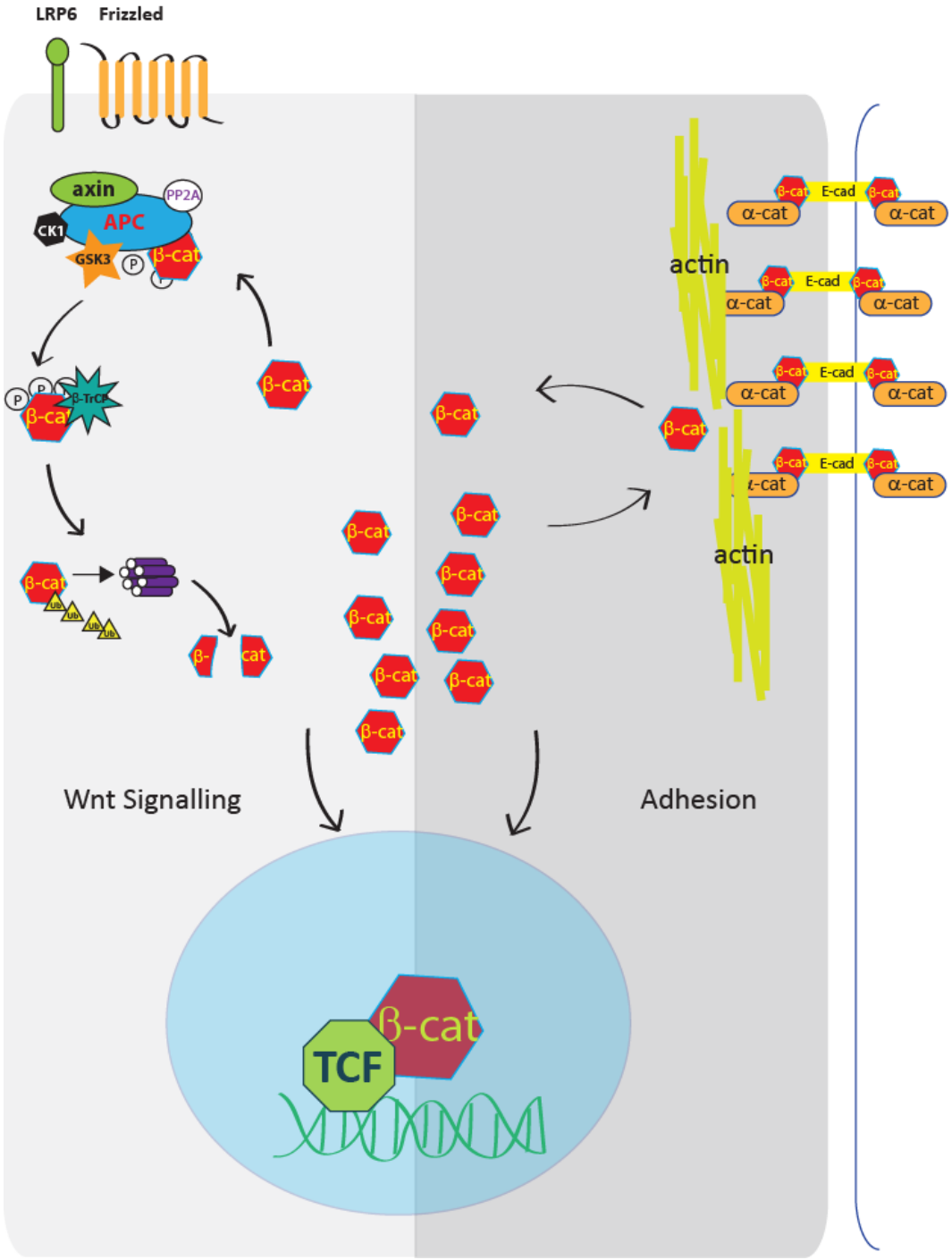



*Figure 1.6: Schematic representation of the dual functions of β-catenin in the Wnt signalling pathway and cell adhesion. β-catenin is the effector protein of the Wnt signalling pathway (left of figure). In the absence of a Wnt signal, β-catenin is marked for degradation by the APC destruction complex prior to degradation by the proteasome. A Wnt signal inhibits the destruction complex and cytoplasmic β-catenin accumulates and can translocate to the nucleus stimulating transcription of Wnt target genes via interaction with TCF/Lef transcription factors. β-Catenin forms a dimeric interaction with α-catenin, which interacts with the actin cytoskeleton. The β-catenin/α-catenin dimer binds to the junctional protein E-cadherin. Both functions draw on the same cellular pool of β-catenin and crosstalk exists between adhesion and Wnt signalling functions.*

### 1.6.3 Role of β-Catenin in Epithelial to Mesenchymal Transition (EMT)

Crosstalk between the adhesion and transcriptional roles of β-catenin has relevance in the process of epithelial to mesenchymal transition (EMT), an important process in the progression of cancer. Tight regulation of cell adhesion is a requirement for normal tissue homeostasis and abnormalities are involved in carcinogenesis through the process of EMT. During EMT, highly organised polarised epithelial cells acquire a phenotype characteristic of more motile mesenchymal cells expressing N-cadherin, with a reduction in E-cadherin (Thiery *et al.* 2009, Zeisberg and Neilson 2009). Wnt target genes under the direct influence of β-catenin are implicated in this process. β-Catenin directly upregulates Snail 2, whereas Wnt signalling-mediated inhibition of GSK3β can cause an increase in the level of Snail 1 (Zhou *et al.* 2004, Yook *et al.* 2006). Both Snail 1 and 2 repress E-cadherin transcription (Barrallo-Gimeno and Nieto 2005). An increase in β-catenin signalling following APC loss in cancer could therefore enhance cancer progression by causing down regulation of E-cadherin in the EMT process. Other Wnt target genes can also promote EMT by either suppression of E-cadherin transcription (Slug) (Vallin *et al.* 2001), up regulation of N-cadherin (Twist) (Howe *et al.* 2003) or cleavage of E-cadherin (ADAM10) (Maretzky *et al.* 2005) (Figure 1.7).

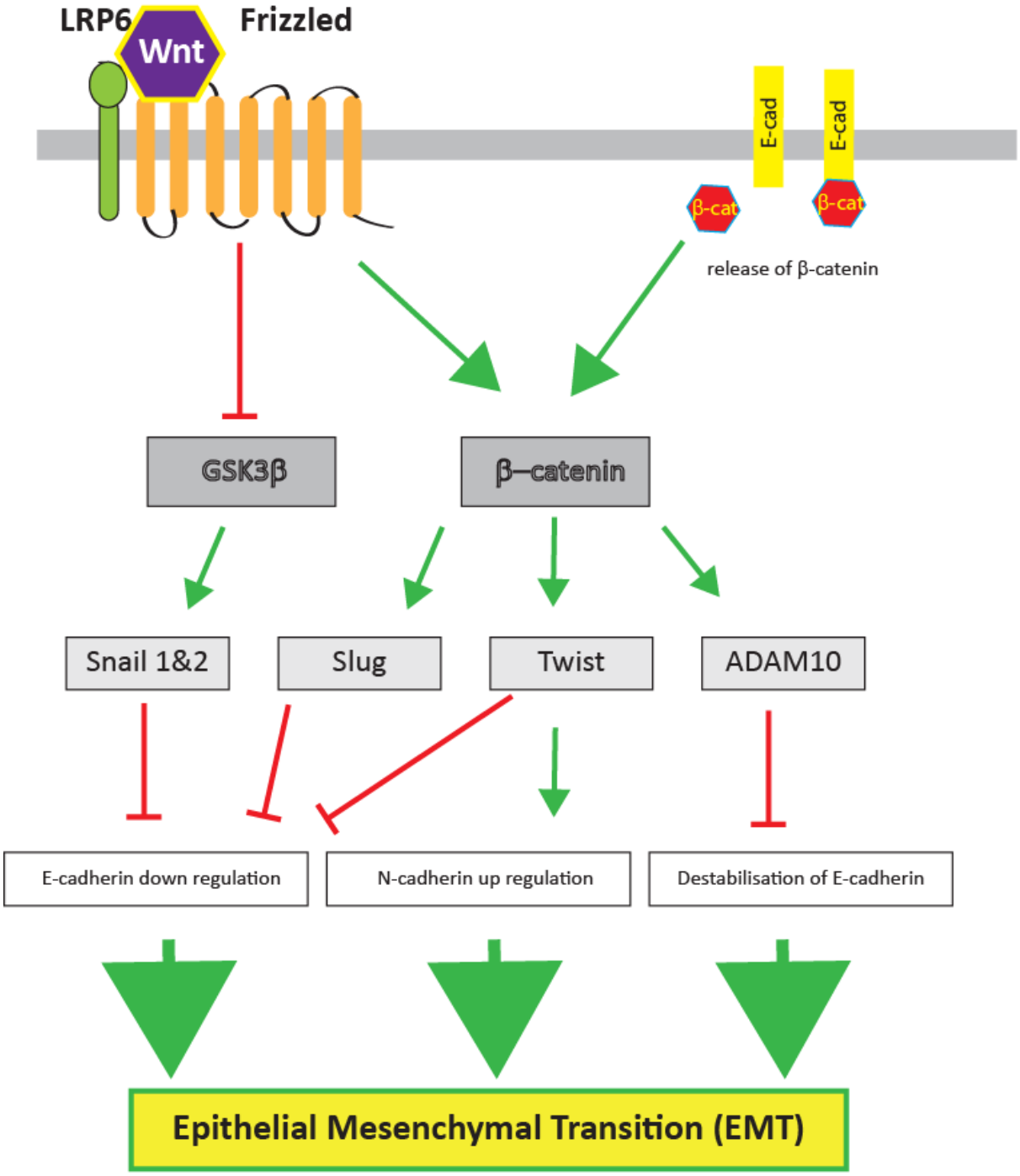



*Figure 1.7: Schematic representation of the involvement of β-catenin in EMT. Wnt signalling and destabilisation of E-cadherin can increase the signalling function of β-catenin. This can lead to up regulation of E-cadherin transcriptional repressors Snail, Slug and Twist, which depress transcription of E-cadherin. EMT is also associated with an up regulation of N-cadherin and cleavage of E-cadherin by ADAM10.*

It is clear that the regulation of β-catenin by two distinct complexes containing either APC or E-cadherin is relevant for the initiation and progression of colorectal cancer. One of the main aims of my thesis is to understand how this is controlled. Mutations in β-catenin or in APC are

associated with cancer, although the frequencies of these defects are different and they do not co-exist. It is unclear how mutations leading to seemingly comparable molecular defects, i.e. stabilised β-catenin, can diverge in terms of disease progression. Determination of how these mutations upset the balance of interactions between β-catenin and APC or E-cadherin may clarify the functional defects that influence cancer progression.

## 1.7 PTEN

### 1.7.1 PTEN and colorectal carcinoma

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumour suppressor gene, located at chromosome 10q23. PTEN mutation is implicated in PTEN hamartomatous tumour syndrome, an autosomal dominant condition that predisposes to colorectal cancer. This is comprised of patients with either Cowden Syndrome or Bannayan–Riley–Ruvalcaba syndrome (Campos *et al.* 2015). Both syndromes carry risk of gastrointestinal polyps with malignant potential as well as extra intestinal manifestations. As many as 80% of patients with a PTEN mutation in Cowden's syndrome will have intestinal polyps, with 18% developing colorectal cancer by age 60 (Nieuwenhuis *et al.* 2012). These patients also carry a risk of developing carcinoma in other organs, including breast, thyroid, endometrium and kidney (Tan *et al.* 2012).

PTEN protein loss is also implicated in sporadic colorectal cancers. The frequency of reported loss varies between studies from 12–78% of cases (Parsons *et al.* 2005, Sartore-Bianchi *et al.* 2009, Wiesweg *et al.* 2013, Yip *et al.* 2013, Chong *et al.* 2014). PTEN mutation has also been linked with the alternative serrated pathway of colorectal carcinogenesis. A large study examining 1,093 colorectal cancers identified PTEN mutation in 5.8% of tumours. Interestingly, these PTEN mutant tumours more commonly had BRAF mutation, MSI high, CIMP high and a proximal location. These are all features associated with sessile serrated tumours (Day *et al.* 2013).

### 1.7.2 PTEN structure and function

The PTEN gene encodes a 403 amino acid protein with both lipid and protein phosphatase activity. It is composed of a phosphatase domain and a C2 domain, which constitute the main functional units of the protein. In addition, the N-terminus has a binding region for phosphatidylinositol (PI) 4,5-bisphosphonate (PIP2), whereas the C-terminal end of the protein has a PDZ binding domain as well as a PEST sequence (Lee *et al.* 1999).

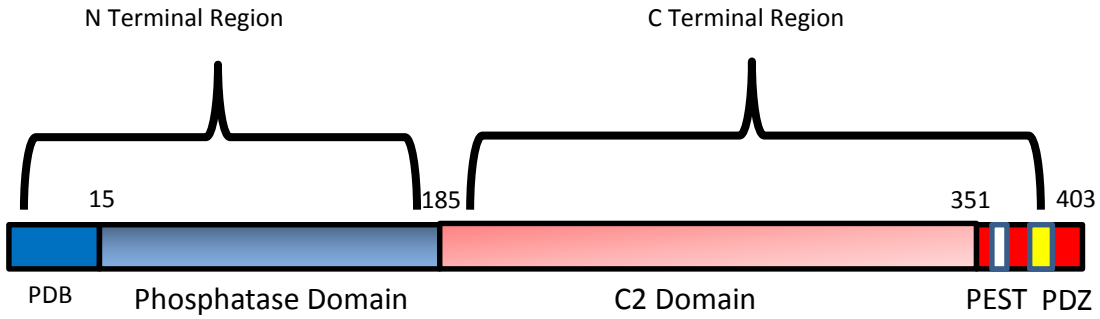



*Figure 1.8: The structure of PTEN protein. The 403 amino acid sequence is composed of 185 N-terminal amino acids containing a PIP2 binding domain between residues 1–15 and a phosphatase domain (residues 25–185). A C-terminus, containing PEST motifs and a PDZ binding region at the C-terminal extreme is joined by the C2 domain (amino acids 185–351) to complete the C-terminal region.*

PTEN is a multifunctional protein with an important role as a tumour suppressor. It plays a key role in the negative regulation of the phosphatidylinositol (PI) 3, 4, 5-triphosphate (PIP3)-mediated activation of the Akt signalling pathway. Ligand activation of receptor tyrosine kinases leads to phosphorylation of PIP2 by phosphoinositide 3-kinase (PIK3) creating PIP3. This activates the Akt pathway, stimulating cell proliferation, progression of the cell cycle, growth and survival (Koul *et al.* 2002, Fata *et al.* 2012, Song *et al.* 2012). PTEN's lipid phosphatase activity directly antagonises PIK3 activity by dephosphorylation of PIP3. This PTEN-induced negative regulation of PIP3 and its downstream effectors can induce a tumour-suppressing effect. PTEN loss on the other hand can result in unrestrained activation of PI3K effectors (Di Cristofano and Pandolfi 2000, Cully *et al.* 2006) (Figure 1.9 h). PTEN mutation is commonly implicated in

prostate and endometrial carcinoma. Loss of the negative regulation of the Akt pathway is thought to account for tumour genesis following PTEN loss in these cancers (Kwak *et al.* 2013)

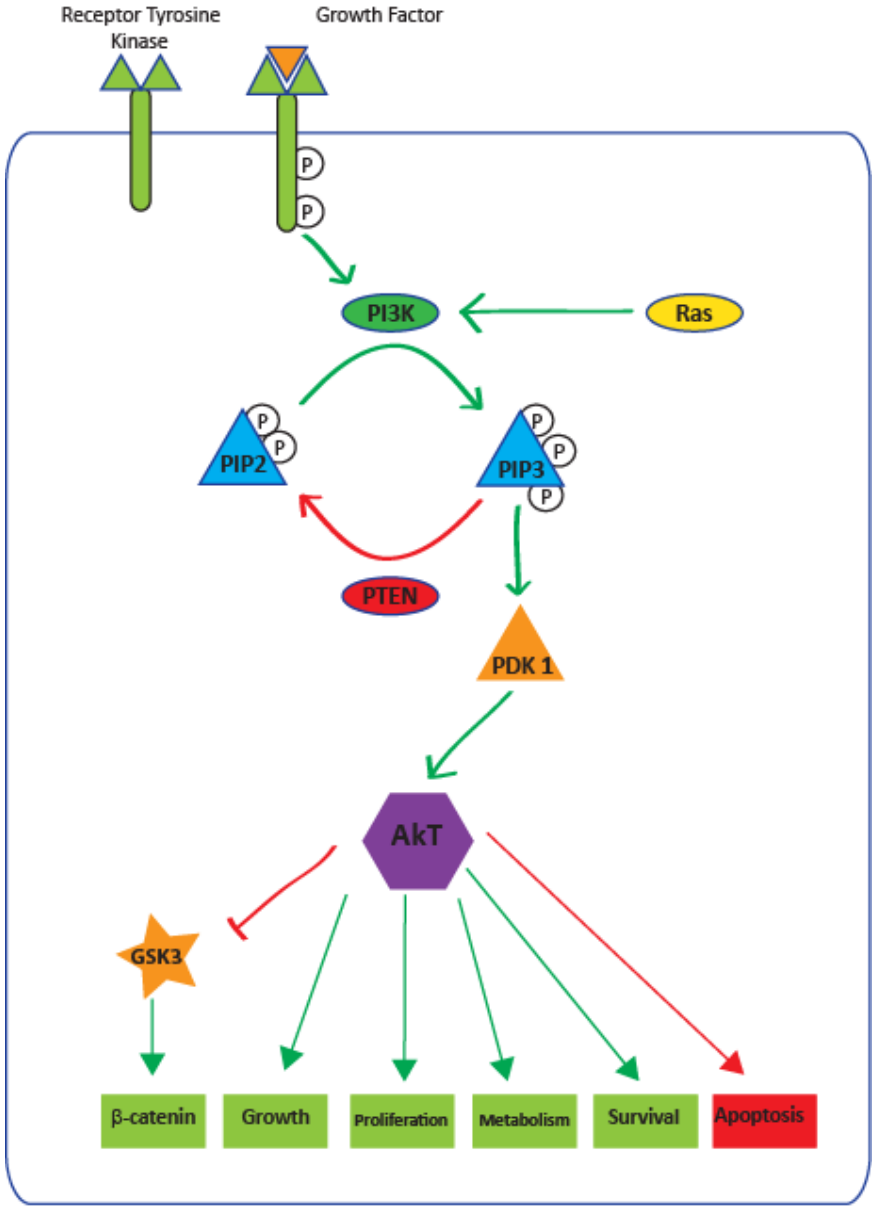



*Figure 1.9: A simplified schematic of PTEN involvement in the PI3K/Akt pathway. Binding of growth factor to tyrosine kinase receptor activates PI3K, which phosphorylates PIP2 to PIP3. The phosphatase activity of PTEN facilitates the reverse conversion of PIP3 to PIP2. PIP3 can then activate Akt via PDK1. Akt has multiple downstream effects. It positively influences cell growth, survival and proliferation while negatively impacting on apoptosis. It also inhibits GSK3$\beta$. This can lead to reduced function of the APC destruction complex resulting in increased levels of $\beta$-catenin. Loss of PTEN leads to activation of the Akt pathway. Ras mutations also feed into this pathway by influencing the function of PI3K.*

Mutations affecting the C-terminal domain can also influence PTEN function and account for the majority of PTEN tumorigenic mutations. Aprroximately 40% of PTEN mutations are located in

exon 5 which contains the phosphatase domains, essential for the lipid and phosphatase functions of PTEN. These mutations result in truncation leading to loss of the phosphatase domain or missense mutations in the phosphatase motif leading to loss of function.(Waite and Eng 2002, Sansal and Sellers 2004). The C2 domain has been shown to play a role in cell migration and EMT in multiple systems. Loss of PTEN can result in increased cell migration, and this has been shown to be specifically related to loss of its protein phosphatase function (Leslie *et al.* 2007). The role of PTEN in the EMT process may be related to an association with adherens junctions.

**1.8 PhD Proposal**

Work in the Näthke laboratory is focused on cell biology of the gut, specifically related to the function of APC and colorectal cancer. In this proposal, I aim to establish factors that regulate the distribution of β-catenin between complexes containing APC and complexes containing E-cadherin.

Specifically, I will:

Establish how a stabilising mutation in β-catenin affects its involvement in complexes with either APC or E-cadherin.

Determine how the absence of PTEN influences the levels of β-catenin, APC and E-cadherin and how it affects the relative interaction of β-catenin with either APC or E-cadherin.

Establish how genetic or biochemical manipulation of β-catenin, APC and PTEN affects the proliferation, migration and invasion of colorectal cancer cells.

Determine if the expression patterns of β-catenin, E-cadherin and PTEN differ between conventional colorectal polyps and polyps with a serrated histology.

Findings from this study will add to our knowledge of how mutations in β-catenin and loss of APC or PTEN change the relative interactions and functional output of β-catenin. This may reveal reasons why tumours characterised by certain mutational signatures progress, while others with related but distinct mutations, may not. Insight into this could highlight opportunities for identifying high-risk patients that justify a more aggressive treatment or surveillance regime with the aim of improving prognosis.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Primary Antibodies

| Antibody | Source | Cat. No. | Clonality | Species | Application | Dilution (Biochemistry) | Dilution (Imaging) |
|---|---|---|---|---|---|---|---|
| β-catenin | Abcam | AB16051 | Monoclonal | Mouse | Biochemistry / Imaging | 1:500 | 1:500 |
| β-catenin 74 | Näthke Self-Generated | N/A | Polyclonal | Rabbit (C-termnus) | Biochemistry | 1:500 | 1:500 |
| M-APC | Näthke Self-Generated | N/A | Monoclonal | Mouse (aa 788-1038) | Imaging | N/A | 1:200 |
| N-APC | Näthke Self-Generated | N/A | Polyclonal | Rabbit (aa 1-300) | Biochemistry | 1:500 | N/A |
| APC–ALI | Näthke Self-Generated | N/A | Monoclonal | Mouse (aa135-422) | Biochemistry | 1:500 | N/A |
| E-cadherin | Cell Signalling | 24E10 | Polyclonal | Rabbit | Biochemistry / Imaging | 1:500 | 1:100 |
| PTEN | Cell Signalling | 9559 | Monoclonal | Rabbit | Biochemistry | 1:500 | N/A |
| PTEN | Sigma Aldrich | Ab-370 | Polyclonal | Rabbit | Imaging | N/A | 1:50 |
| Phospho-Akt | Cell Signalling | 4060 | Monoclonal | Rabbit | Biochemistry | 1:500 | N/A |
| SNAIL | Cell Signalling | #3879 | Monoclonal | Rabbit | Biochemistry | 1:50 | N/A |
| GAPDH | Life Technologies | MA5-15738 | Monoclonal | Mouse | Biochemistry | 1:1000 | N/A |
| Rhodamine phalloidin | Molecular Probes | T-7471 | N/A | N/A | Imaging | N/A | 1:50 |

### 2.1.2 Secondary antibodies and nuclear stain

All secondary antibodies were provided by molecular probes (Invitrogen, Oregon USA). The nuclear stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was sourced from Sigma-Aldrich (D9542).

### 2.1.3 Short interfering RNAs

| Product | Company | Symbol | Modification | Target Sequence |
|---|---|---|---|---|
| β-catenin siRNA | Qiagen | CTNNB1 | nil | Sense 5′-CGGGAUGUUCACAACCGAATT-3′ Anti-sense 5′-UUCGGUUGUGAACAUCCCGAG-3′ |
| β-catenin siRNA (Fluorescent tag) | Qiagen | CTNNB1 | 3′Cy5 | Sense 5′-CGGGAUGUUCACAACCGAATT-3′ Anti-sense 5′-UUCGGUUGUGAACAUCCCGAG-3′ |
| E-cadherin siRNA | Qiagen | CDH1 | nil | Sense 5′-AGGUAUUGUCUACUCUGAATT-3′ Anti-sense 5′-UUCAGAGUAGACAAUACCUAG-3′ |
| E-cadherin siRNA (Fluorescent tag) | Qiagen | CDH1 | 3′Cy5 | Sense 5′-AGGUAUUGUCUACUCUGAATT-3′ Anti-sense 5′-UUCAGAGUAGACAAUACCUAG-3′ |

| | | | | |
|---|---|---|---|---|
| PTEN 3 siRNA | Qiagen | PTEN | nil | Sense<br>5′-ACGGGAAGACAAGTTCATGTA-3′<br>Anti-sense<br>5′-UACAUGAACUUGUCUUCCGT-3′ |
| PTEN 8 siRNA | Qiagen | PTEN | nil | Sense<br>5′-CGAUAGCAUUUGCAGUAUATT-3′<br>Anti-sense<br>5′-UAUACUGCAAAUGCUAUCGAT-3′ |
| α-catenin 1 siRNA (Fluorescent tag) | Qiagen | CTNNA1 | 3′Cy5 | Sense<br>5′-GUGGAUAAGCUGAACAUUATT-3′<br>Anti-sense<br>5′-UAAUGUUCAGCUUAUCCACTT-3′ |
| α-catenin 2 siRNA (Fluorescent tag) | Qiagen | CTNNA2 | 3′Cy5 | Sense<br>5′-CCAUUGUAUUCACUAACUATT-3′<br>Anti-sense<br>5′-UAGUUAGUGAAUACAAUGGTA-3′ |
| APC SiRNA DUPLEX 5 | Sigma | APC | NIL | Sense<br>5′-GAUGAUAUGUCGCGAACUUU-3′ |

| | | | | |
|---|---|---|---|---|
| | | | | Anti-sense<br>5′-AAGUUCGCGACAUAUCAUCUU-3′ |
| APC SiRNA DUPLEX 6 | Sigma | APC | NIL | Sense<br>5′-GAGAAUACGUCCACACCUUUU-3′<br>Anti-sense<br>5′-AAGGUGUGGACGUAUUCUCUU-3′ |
| APC SiRNA DUPLEX 7 | Sigma | APC | NIL | Sense<br>5′-GAACUAGAUACACCAAUAAUU-3′<br>Anti-sense<br>5′-UUAUUGGUGUAUCUAGUUCUU-3′ |
| APC SiRNA DUPLEX 5 (Fluorescent tag) | Sigma | APC | 3′Cy5 | Sense<br>5′-GAUGAUAUGUCGCGAACUUU-3′<br>Anti-sense<br>5′-AAGUUCGCGACAUAUCAUCUU-3′ |
| APC SiRNA DUPLEX 6 (Fluorescent tag) | Sigma | APC | 3′Cy5 | Sense<br>5′-GAGAAUACGUCCACACCUUUU-3′<br>Anti-sense<br>5′-AAGGUGUGGACGUAUUCUCUU-3′ |

| | | | | |
|---|---|---|---|---|
| APC SiRNA DUPLEX 7 (Fluorescent tag) | Sigma | APC | 3′Cy5 | Sense 5′-GAACUAGAUACACCAAUAAUU-3′ Anti-sense 5′-UUAUUGGUGUAUCUAGUUCUU-3′ |
| Negative Control | Qiagen | | | All stars negative control Flexitube siRNA (20mmol) Product number:1027418 |

### 2.1.4 qPCR primers

| Gene | Company | 5′ forward primer 3′ | 5′ reverse primer 3′ | TM (°C) |
|---|---|---|---|---|
| Actin | Eurofins | CTGGGAGTGGGTGGAGGC | TCAACTGGTCTCAAGTCA | 59.3 |
| AXIN2 | Eurofins | TGGCTATGTCTTTGCACCAG | TGTTTCTTACTGCCCACACG | 59.3 |
| β-catenin | Eurofins | ATGGCTTGGAATGAGACTGC | TTCCATCATGGGGTCCATAC | 59.3 |
| E-cadherin | Eurofins | TGGAGGAATTCTTGCTTTGC | CGTACATGTCAGCCAGCTTC | 59.3 |

### 2.1.5 Cell lines

Cell lines were supplied by Horizon Discovery Ltd. Four HCT116 human colorectal cancer cell lines were studied. These cell lines differed in their β-catenin proteins. The parental cell line is heterozygous for β-catenin (Δ45/+) and has one wild-type β-catenin allele and a corresponding mutant β-catenin allele that lacks serine 45. This residue is a target for phosphorylation by the APC destruction complex. Without phosphorylation of serine 45, subsequent phosphorylations at residues 41, 37 and 33 cannot take place. As a result, β-catenin cannot be degraded. This parental cell line was used to generate two further cell lines differing in their expression of β-catenin. This was done by insertion of Lox P sites and the Cre recombinase system.

The parental cell line has undergone heterozygous deletion of either the mutant or wild type β-catenin to generate two further cell lines.

The cell line referred to as “mutant” has had the wild type β-catenin deleted so exclusively expresses the mutant β-catenin (Δ45/-). Throughout this thesis I will refer to this cell line as “mutant” to reflect the fact that it only expresses mutant (Δ45) β-catenin.

The cell line referred to as “wild type” only expresses the wild-type β-catenin protein (-/+). It has had the mutant (Δ45) deleted, leaving only wild type β-catenin. Throughout the thesis this cell line will be referred to as wild type to reflect the fact that it only expresses one normal (wild type) β-catenin protein.

The three cell lines described above all have wild type APC, E-cadherin and PTEN.

The PTEN (-/-) cells are characterised by the same β-catenin expression as the parental cell line, but differ in that it has a homozygous deletion of PTEN. All of these cell lines carry an endogenous KRAS mutation (KRAS G13D/+). The PTEN (-/-) cells also have wild type APC and E-cadherin.

## 2.2 Methods

### 2.2.1 Cell Culture

#### 2.2.1.1 Thawing of cells

Cells were removed from cold storage in liquid nitrogen. A T75 tissue culture flask was prepared by addition of 15 ml of growth media. The vial containing the frozen cell pellet was immersed in a water bath at 37 °C briefly. Upon removal, 1 ml of prewarmed growth media was added to the vial and gently pipetted up and down. The media containing the cells were then added to the tissue culture flask.

#### 2.2.1.2 Cell passaging

HCT116 human colorectal carcinoma cell lines were maintained in RPMI growth media supplemented with 10% FBS and penicillin/streptomycin under sterile conditions in tissue culture flasks. Cells were inspected daily. When cells had reached 70–80% confluence, they were passaged in the tissue culture hood. The cells were washed once in 1% trypsin. 4 ml of trypsin was then added to the flask and the flask returned to the incubator at 37 °C. After approximately 5 minutes the cells were inspected for detachment from the culture vessel. 8 ml of culture media was added to the flask and an appropriate volume of this mixture was added to a new tissue culture vessel at an appropriate density as experiments dictated.

#### 2.2.1.3 Cell counting

A haemocytometer and cover slip were cleaned with 70% ethanol and left to dry in a tissue culture hood. A small volume of trypsinised cells in growth media was pipetted under the cover slip. Cells were visualised using a 20× microscope objective lens. Cells were counted in each of four quadrants and a mean count determined. This value was then used to determine the appropriate volume of cells required for each specified procedure.

#### 2.2.1.4 Cryopreservation of cells

Cells were trypsinised and 8 ml of growth media was added. The mixture was pipetted into a falcon tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was aspirated and 1 ml of freezing media (90% FBS, 10% DMSO) was added. The cells were resuspended and 100 μl aliquots were added to cryovials. The vials were stored in a cryofreezing chamber at –80 °C for one night before being transferred to liquid nitrogen for long-term storage.

### 2.2.2 Cell siRNA transfection

siRNA transfections were carried out in T25 tissue-culture flasks. Cells were seeded the day prior to transfection to ensure they had reached a confluence of approximately 50% at the time of the transfection procedure. For each siRNA used, 8 μl of 10 μM siRNA duplex was added to 200 μl of Opti-MEM and flick mixed. 16 μl of INTERFERin™ (Polyplus Transfection™) was added to the mixture and homogenised for 10 seconds using a vortex. The same volumes were used for each siRNA duplex with the exception of APC in which 16 μl of 10 μM siRNA, 32 μl INTERFERin and 200 μl of optimum were used.

The mixtures were incubated for 10 minutes at room temperature. During the incubation period, growth medium was removed from each tissue culture flask and replaced with fresh prewarmed growth media. 200 μl of the transfection mixture was added to each tissue culture flask. Cells

were incubated at 37 °C for 24 hours, at which point the transfection procedure was repeated as described above. A further 24 hour period of incubation was performed prior to cell harvesting.

siRNA was chosen to perform down knock downs due to the reliability and reproducibility of results. In addition the system is straight forward to replicate in multiple experiments and fitted in well with the time constraints of the project.

For each experiment involving targeted depletions using siRNA against specific genes negative control experiments were carried out in tandem. The control experiments were treated with scrambled siRNA. All other reagents used in the experimental conditions (interferin and optimum) were also used to treat cells under control conditions. This approach was taken whether the cells were to be used in protein experiments, RNA experiments or imaging.

### 2.2.3 Microscopy

#### 2.2.3.1 Collagen coating of coverslips

Cover slips were sterilised in 70% ethanol then each one placed in a well of a six-well plate prior to washing twice in PBS. 2 ml of collagen was added to the well and left for 4 hours in a sterile tissue culture hood. The collagen was aspirated from the well and the plate was left to incubate overnight under ultraviolet light in a tissue culture hood.

#### 2.2.3.2 Staining of cells

Five hundred thousand cells were seeded onto collagen-coated cover slips and incubated at 37 °C for 48 hours to achieve a confluence of approximately 80%. Growth medium was aspirated and cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes, which was prepared prior to the procedure. Specifically, 1.85 g of PFA was dissolved in 3.7 ml doubly distilled $H_2O$ and 100 µl 1 M potassium hydroxide was added. The solution was placed in a water bath at 60 °C. When

fully dissolved, the pH was adjusted to 7 and diluted to a final concentration of 4% in 1× PHEM buffer (60 mM Pipes, 4 mM $MgSO_4$ 25 mM (Formedium, Hepes 10), 10 mM EGTA, pH 6.9) and warmed to 37 °C prior to use. After fixation, cells were washed in wash buffer (0.02% sodium azide, 0.2% BSA in PBS). Cover slips were then transferred onto a parafilm sheet inside a dark humidified chamber for the remainder of the protocol.

Cells were permeabilised in permeabilisation buffer (1% NP-40 in phosphate-buffered saline (PBS) for 10 minutes before repeated washing in wash buffer. Blocking buffer (5% normal goat serum, 2% bovine serum albumin (BSA), 0.02% sodium azide and 0.1% TX-100 in PBS) was added to coverslips for 30 minutes prior to a further wash.

Primary antibodies were diluted at the appropriate concentration in incubation buffer (2% BSA, 0.02% sodium azide, 0.1% TX-100 in PBS) and centrifuged for 5 minutes at 13,000*g* to remove aggregates before addition of 150 µl to each cover slip. Cells were incubated in primary antibody for 1 hour in the dark at 37 °C then washed in wash buffer for 3 × 5 minutes.

Secondary antibodies attached to a fluorescent probe (Alexa Fluor 488, Alexa Fluor 568 or Alexa Fluor 647) (Molecular Probes, Invitrogen) were diluted in incubation buffer, centrifuged for 5 minutes at 13,000*g* before addition of 150 µl to each cover slip and incubation in the dark for 1 hour at 37 °C before washing in wash buffer for 3 × 5 minutes.

Cells were then counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1 µg/ml) for 10 minutes followed by a further wash step. Cover slips were tilted to allow drying and were then inverted and placed on a drop of mountant (deoxygenated 90% v/v glycerol, 20 mM Tris pH 8.8, *p*-phenylenediamine). Coverslip edges were sealed with nail polish and left to dry in the dark for 30 minutes prior to storage at –20 °C. Coverslips were thawed at room temperature and washed with ultra-pure water prior to imaging.

#### 2.2.3.3 Staining of mouse tissue

Tissue sections were placed in a slide holder at room temperature and rinsed twice in PBS for 10 minutes prior to permeabilisation with 1% Triton for 20 minutes followed by a further PBS rinse.

Sections were then blocked with blocking buffer (5% normal goat serum, 2% BSA, 0.02% sodium azide, 0.1% TX-100 in PBS) for 2 hours. They were incubated overnight with primary antibodies at 4 °C in working buffer (WB) (0.3% normal goat serum, 0.1% BSA, 0.2% TX-100 in PBS pH 7.4).

Sections were then washed in WB five times for 5 minutes. Sections were then incubated in secondary antibodies diluted at a concentration of 1:500 for 1 hour at room temperature.

The sections were then rinsed in WB for 5 × 5 minutes then 3 × 5 minutes in PBS prior to counterstaining with DAPI at 10 µg/ml (1:50) then again rinsed in PBS.

A small drop (10 µl) of mountant (Prolong gold) was placed on top of the tissue section and a number 1.5 22 × 22 mm coverslip was placed on top. These were allowed to harden at room temperature overnight and then sealed with nail polish the following day. Coverslips were washed with ultra-pure water prior to imaging. Samples were then stored at –20 °C.

#### 2.2.3.4 Staining of human tissue

The same procedure described above was followed for staining human tissue slides.

#### 2.2.3.5 Confocal microscopy

High-resolution images were captured using a Zeiss 710 confocal microscope. Images were acquired at 0.2 µm *z*-section intervals using a 40× objective lens. Images were processed using

the Zenn software imaging system. Further analysis was carried out using imageJ (Image processing and analysis in JAVA) and Imaris (Bitplane, Oxford Instruments).

### 2.2.4 Protein Analysis

#### 2.2.4.1 Cell lysis

Cells were placed on ice for 5 minutes. Media was aspirated and the cells were washed three times with chilled PBS. Proteases; LPC (leupeptin, pepstatin A & chymostatin, each at 10 mg/ml in DMSO), sodium orthovanadate (0.1 M) and sodium fluoride (1 M) were added to MEBC lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM NaEDTA, 5 mM NaEGTA, 40 mM β-glycerophosphate and 0.5% NP-40) at a concentration of 1:100 (10 μg/ml). 100 μl of the mixture was spread over the base of a T25 flask and left on ice for 10 minutes. If the lysate was intended for incubation with phospho-specific antibodies, the same procedure was followed but with an alternative incubation buffer. In these cases the cells were incubated in (1× lysis buffer, 270 mM sucrose, 1 mM benzamide, 0.2 mM PMSF, 1 mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 0.1 μM microcystin LR, 1 tablet protease inhibitor and 4.2 ml deionised $H_2O$).

Material was then scraped from the dish and pipetted into a 1.5 ml Eppendorf tube. The tube was centrifuged for 20 minutes at 14,000 rpm at 4 °C. An 8 μl aliquot was taken for determination of protein concentration while the remainder was snap frozen in liquid nitrogen, as was the pellet. Samples were stored at –80 °C.

#### 2.2.4.2 Protein concentration in lysates

The Bradford assay was used to determine the protein concentration of cell lysate. Stock standards used were 0, 0.25, 0.5, 1, 1.5 and 2 mg/ml. Each standard was produced by adding 10 μl of the relevant stock standard to 790 μl of water and 200 μl of Bradford reagent.

Protein samples were measured in triplicate. 2 μl of each sample was added to 798 μl of water and 200 μl of Bradford reagent. Samples were vortexed prior to pipetting into a cuvette. They were then analysed using an OD595 spectrophotometer.

**2.2.4.3 Cell fractionation**

The following cell fractionation protocol utilises a ProteoExtract® Subcellular Proteome Extraction Kit (EMD Biosciences). Cells were seeded at a density of 50% in a T25 flask and incubated at 37 °C under sterile conditions for 48 hours to achieve a cell density of 80–90% confluence. Buffers I–III and Benzonase® were kept on ice while Buffer IV and the protease inhibitor cocktail were stored at room temperature. Prior to extraction, buffers were mixed by vortexing.

Growth media was aspirated from the cell culture vessel leaving approximately 500 µl. The mixture was pipetted into a 1.5 ml Eppendorf tube containing 500 µl of ice cold wash buffer. The tube was centrifuged at 300*g* for 10 minutes and the supernatant was carefully aspirated. This wash step was performed once more using 500 µl of wash buffer.

2.5 µl of protease inhibitor was added to 500 µl of extraction buffer I, and flick mixed in an Eppendorf tube. The cell pellet was gently resuspended in this mixture and incubated at 4 °C under gentle agitation for 10 minutes. The tube was then centrifuged for 10 minutes at 1000*g*. The supernatant was carefully pipetted into another Eppendorf tube as the Fraction 1 (cytoplasmic) fraction and snap frozen in liquid nitrogen.

500 µl of extraction buffer II was mixed with 2.5 µl of Protease Inhibitor cocktail and added immediately to the cell pellet. This was resuspended by gentle pipetting prior to incubation at 4 °C for 30 minutes under gentle agitation. Insoluble material was sedimented by centrifugation at 6000*g* for 10 minutes at 4 °C. The supernatant was transferred to a separate tube as Fraction 2 (Membrane) and snap frozen in liquid nitrogen.

250 µl of extraction buffer III was mixed with 2.5 µl Protease Inhibitor cocktail and 0.75 µl of Benzonase®. This was immediately added to the cell pellet by pipetting up and down. It was then incubated for 10 minutes at 4 °C under gentle agitation. Insoluble material was sedimented by

centrifugation at 6800*g* for 10 minutes at 4 °C. The supernatant was transferred to a separate tube as Fraction 3 (Nucleus) and snap frozen in liquid nitrogen.

250 µl of extraction buffer IV was mixed with 2.5 µl Protease Inhibitor cocktail and immediately added to the cell pellet. Residual particles were suspended by pipetting up and down. This was snap frozen in liquid nitrogen as Fraction 4 (Cytoskeleton). Samples were stored at –80 °C.

### 2.2.5 Western blot

#### 2.2.5.1 SDS PAGE

An appropriate volume of 4× loading buffer, protein sample and $H_2O$ were mixed in an Eppendorf tube. Samples were denatured by boiling for 10 minutes at 99 °C. After boiling, one twentieth the volume of DTT was added to each sample, flick mixed and briefly vortexed. 10 µl of Benchmark protein ladder (Invitrogen 10747012) was added to the first well of the gel. An appropriate volume of sample was added to each well and the gel was run at 90 V in running buffer (outer chamber 475 ml $H_2O$, 25 ml 20X MOPS-SDS, running buffer (FORMEDIUM$^{TM}$ MOPS-SDS0500), inner chamber 190 ml $H_2O$, 10 ml 20X MOPS-SDS running buffer with 500 µl Nupage antioxidant (Invitrogen 162262)) until the gel had reached the bottom of the chamber.

#### 2.2.5.2 **Electrophoretic transfer**

The gels were transferred to a 0.1 µm Protran nitrocellulose membrane using a trans-blot transfer cell. Transfer buffer (200 ml 10× laemmli, 75 ml methanol and 1 ml 20% SDS in 1 L of $H_2O$ was used for transfer, which took place for 16 hours at 30 V. Transfers were carried out at 4 °C.

#### 2.2.5.3 Ponceau detection of proteins

Membranes were submerged in Ponceau S solution to visualise all protein bands. This allowed marking of the protein ladder with a pen and cutting of the membrane as necessary.

#### 2.2.5.4 Detection of transferred proteins

The membrane was washed in wash buffer (1 × TBS, 0.002% Triton X-100) to remove the Ponceau stain. Membranes were then blocked in blocking buffer (5% milk powder, 1 × TBS. 0.02% Triton X-100 and 0.02% sodium azide) for 1 hour under gentle agitation at room temperature. Membranes were then transferred to a vessel containing blocking buffer with the appropriate primary antibody. Incubation time was antibody dependent and carried out at 4 °C.

The membrane was then washed thoroughly for at least 3 × 5 minutes in wash buffer prior to incubation in blocking buffer containing the appropriate secondary antibody. Incubation in the secondary antibody lasted one hour at room temperature. A further 3 × 5 minute wash was carried out prior to imaging and quantification using the LI-COR Odyssey system. Protein signal intensity normalised to an appropriate loading control in the same sample was used to quantitate protein levels.

### 2.2.6 Immunoprecipitation

40 μl of either sepharose protein A or G beads as appropriate were added to a 1.5 ml screw top Eppendorf tube on ice. Beads were washed × 2 in 750 μl of MEBC prior to addition of 5 μg of antibody or control IgG along with 200 μl of MEBC and incubated overnight on a wheel rotator at 4 °C. The tubes were then centrifuged briefly and were again washed twice in MEBC. 50 μg of lysate was kept for a pre-IP sample and 1 mg of lysate was then added to the beads and incubated for 2 hours at 4 °C. The volume of lysate used in the IP was approximately 500–750 μl. The beads were centrifuged briefly and a further 50 μg of lysate was taken for a post-IP

sample. The beads were washed five times in MEBC with LPC, sodium orthovanadate (0.1 mM) and sodium fluoride (0.1 mM) on ice. 20 μl of 1.3 × loading buffer was added to the beads and they were vortexed and snap frozen in liquid nitrogen. Samples were then loaded and separated using SDS PAGE and transferred as described above.

### 2.2.7 Transfection of expression vectors

Cells were grown in a well of a 6-well plate. When they were approximately 60% confluent the transfection procedure was performed. 2 ml of fresh growth media was placed in the well. The transfection mix was then prepared as follows. For each well to be transfected, 1 μg of the appropriate vector was added to 200 μl of optimum transfection media. To this, FuGENE 6 (Promega UK©) transfection reagent was added in a volume exactly double the volume of the vector. For example, if 1 μg of vector equalled 1 μl then 2 μl of FuGENE 6 was used.

The appropriate volume of vector was added to the 200 μl of Opti-MEM and flick mixed. The FuGENE 6 was then added to the mixture before vortexing. This was left to incubate at room temperature for 15 minutes. 200 μl of the transfection mixture was then added dropwise to each well. Cells were incubated at 37 °C and harvested after 48 hours. The PTEN vectors were a kind gift from Dr Nick Leslie, Heriot Watt University, Edinburgh, Scotland. β-Catenin constructs encoding wild-type β-catenin protein and a mutant β-catenin with the deletion of serine residue 45 were used. Both had a myc tag. These constructs were available in-house and were originally developed by Dr Rolf Kemler described in (Aberle *et al.* 1997).

### 2.2.8 Flow Cytometry

#### 2.2.8.1 FACS assisted cell counting

Adherent cells were washed twice using PBS. Cells were then trypsinised and incubated at 37 °C until detached from the culture vessel. Trypsin was deactivated by addition of growth media and

cells were transferred to a Falcon tube. DAPI was diluted at a concentration of 1:500 in PBS and 630 μl was added to a FACS tube. The cell containing mixture was flick mixed and 70 μl added to the PBS/DAPI solution. Cells were counted using a Verse flow cytometer.

#### 2.2.8.2 FACS staining

Cells were suspended at approximately $5 \times 10^5$ cells/ml in PBS and washed twice in PBS + 1% w/v BSA (1 g/100 ml PBS) and pelleted by centrifugation at 2000 rpm for 5 minutes prior to aspiration of the supernatant. The cells were then resuspended in 500 μl of staining buffer (50 μg/ml of propidium iodide and 50 μg/ml ribonuclease A in PBS + 1% BSA) The cells were then incubated at room temperature, protected from light for 20 minutes prior to cell cycle assessment using a Verse flow cytometer.

### 2.2.9 Cell Function Assays

#### 2.2.9.1 Cell proliferation assay

Cells were counted using a Verse flow cytometer. Two thousand cells were seeded per well of a 96-well plate. Each well was imaged once every 24 hours using the Incucyte Zoom live cell imaging system (Essen Bioscience). Cell confluence was calculated using the automated Incucyte zoom image analysis software.

#### 2.2.9.2 Cell migration assay

Fifty thousand cells were seeded per well of a 96-well ImageLock™ plate and incubated at 37 °C for 24 hours achieving a confluence of 90–100%. A wound 700–800 μm wide was created in each well using the Essen Bioscience WoundMaker™. Each well was washed ×3 in PBS prior to addition of 100 μl of growth media. Plates were incubated at 37 °C in the Incucyte Zoom live cell imaging system. Each well was imaged at two hourly intervals for 48 hours. Relative wound density was calculated using the Cell migration analysis software platform (Essen Bioscience).

#### 2.2.9.3 Cell Invasion assay

Fifty thousand cells were seeded per well of a 96 well ImageLock™ plate and incubated at 37 °C for 24 hours achieving a confluence of 90–100%. A wound 700–800 μm wide was created in each well using the Essen Bioscience WoundMaker™. Each well was washed ×3 in PBS prior to addition of 50 μl of matrigel. Plates were placed in an incubator at 37 °C for 30 minutes to allow the matrigel to set. 100 μl of growth media was then added to each well. Plates were incubated at 37 °C in the Incucyte Zoom live cell imaging system. Each well was imaged at two hourly intervals for 48 hours. Relative wound density was calculated using the Cell invasion analysis software platform (Essen Bioscience). The invasion assay differs from the migration assay by the presence of the matrigel. This forms a barrier that the cells have to invade through in order to cross the wound.

### 2.2.10 Quantitative real-time PCR (qPCR)

RNA was isolated from cells using a Nucleospin kit (Machery Nagel) according to the manufacturer's instructions. The concentration of extracted RNA was determined using a NANODROP 1000 spectrophotometer (Thermo Scientific) and 1 μg was used for synthesis of cDNA. A qscript™ cDNA synthesis kit (Quanta Biosciences) was used according to the manufacturer's instructions along with a mastercycler gradient (Eppendorf). 5 μg of cDNA was used for analysis with a CFX connect real-time qPCR system (BIORAD) and PerfeCta SYBR Green FastMix for iQTM (Quanta Biosciences) according to the manufacturer's instructions.

For each cDNA amplification reaction, the time required to reach the exponential phase was normalised to a housekeeping gene (Actin) of the same cDNA sample and calculated relative to the control cell samples. To account for the difference in efficiency of each amplification reaction, the calibration for each qPCR reaction was achieved by measuring the point of temporary exponential DNA amplification in a set of serially diluted cDNA samples. For each

qPCR experiment Actin was used as a reference gene control. All changes in target gene transcript were measured relative to Actin. Actin was unchanged following any of the treatments in any of the cell lines used.

### 2.2.11 Histological assessment of tissue slides

The aim of assessing staining pattern on immuno-histochemistry slides was to determine if the location of β-catenin and E-cadherin differed between polyps of conventional and serrated histology. The cells were divided into three regions, Membrane, cytoplasm and nucleus. A semi-quantitative approach was taken to assessing the slides. The predominant location of β-catenin and E-cadherin was classified as membrane, cytoplasm or nucleus. .Nuclear β-catenin stain was classified as present or absent. To compare stain intensity of PTEN between areas of conventional and serrated histology a score of low, medium or high was applied to indicate stain intensity. Slides were scored in conjunction with a consultant pathologist. A semi-quantitative approach to classification of signal intensity from immuno-histochemical slides in colorectal cancer is a recognised approach (Brown *et al.* 2014) (Huels et al. 2015).

### 2.2.12 Statistical Analysis

Unless otherwise stated t-test was undertaken to determine statistical significance. Significance level was set at $p<0.05$. Statistical values are only quoted where the result is discussed in the text. Results that are not addressed by further discussion will not have a quoted p value applied to them.

# 3 Point Mutation in β-Catenin Stabilises It and Affects Its Interaction with APC and E-cadherin

## 3.1 Introduction

β-Catenin is a multifunctional protein, with roles in cell adhesion and in transcriptional regulation responsive to Wnt signalling (Figure 1.6). Function in the Wnt pathway is regulated by APC. The role of β-catenin in adhesion is dependent on the interaction with E-cadherin to form functional adhesion junctions. Both APC and E-cadherin compete for overlapping binding regions on β-catenin, so their binding is mutually exclusive. However, adhesive and transcriptional functions have been shown to rely on the same pool of β-catenin.

Overexpression of E-cadherin can reduce the contribution of β-catenin to the Wnt signalling pathway in both *Xenopus* (Heasman *et al.* 1994) and *Drosophila* (Sanson *et al.* 1996), whereas a reduction in E-cadherin expression can augment the signalling function of β-catenin (Cox *et al.* 1996). A reduction in Wnt target gene expression and the translocation of β-catenin from the cell nucleus to the membrane can be achieved by expression of cadherin in SW480 colorectal cancer cells (Sadot *et al.* 1998).

Loss of APC, on the other hand, results in increased β-catenin due to loss of a degradative function with subsequent translocation to the cell nucleus and transcription of Wnt target genes (He *et al.* 1998, van de Wetering *et al.* 2002, Sansom *et al.* 2004). This process is implicated as an early and very common event in colorectal cancer (Miyaki *et al.* 1994, Fodde 2002).

Mutations in β-catenin may also influence its ability to function in either a signalling or adhesive role. A study in human colorectal cancer cells (HCT116) found that exclusive expression of a mutant-stabilised β-catenin decreased the strength of the bonds with E-cadherin compared with cells with only wild-type β-catenin. Single cells were brought into contact very briefly (1–300 ms)

then one was pulled away using a cantilever. Bond strength was measured using atomic force microscopy. Cells with one wild type and one mutant protein also exhibited a weaker E-cadherin–cadherin bond than cells with only wild-type β-catenin. This signifies that the mutant β-catenin may act in a dominant negative fashion whereby the presence of mutant β-catenin can impact negatively on bond strength despite the presence of the wild type β-catenin protein.. Comparable amounts of E-cadherin were, however, identified in association with β-catenin. The effects of the mutations on interaction with APC were not assessed (Bajpai *et al.* 2013). A decrease in the association between N-terminally deplete stabilised β-catenin and E-cadherin has been identified previously, but again no assessment of the interaction with APC was made (Chan *et al.* 2002).

It is clear that the regulation of β-catenin in complexes with either APC or E-cadherin is central to the balance of its function in cell adhesion and transcriptional regulation. Disruption of these interactions is critical in the pathogenesis of colorectal cancer. I aim to explore the factors regulating these β-catenin complexes and the consequences of a point mutation leading to stabilisation of β-catenin.

Specifically I will:

1) Compare total protein levels of APC, β-catenin and E-cadherin in parental, mutant and wild-type HCT116 cells.
2) Measure transcript levels of β-catenin, E-cadherin and AXIN2 in these three cell lines.
3) Establish where in the cell β-catenin is located and if this changes in response to depletion of APC, β-catenin, E-cadherin or PTEN.
4) Determine how stabilised β-catenin affects the relative level of interaction between β-catenin in complexes containing APC and those containing E-cadherin.
5) Establish if overexpression of β-catenin can cause changes in protein level of APC or E-cadherin or affect transcription of AXIN2 and E-cadherin.

## 3.2 Results

### 3.2.1.1 Stabilising β-catenin affects the abundance of APC, β-catenin and E-cadherin in HCT116 cells

I determined the total protein levels of APC, β-catenin and E-cadherin in the three HCT116 colorectal cancer cell lines, defined in Material and Methods (Parental (Δ45/+), mutant (Δ45/-) and wild type (-/+)).

Cell lysis and western blot analyses showed differential expression of APC, β-catenin and E-cadherin in the cell lines studied (Figure 3.1).

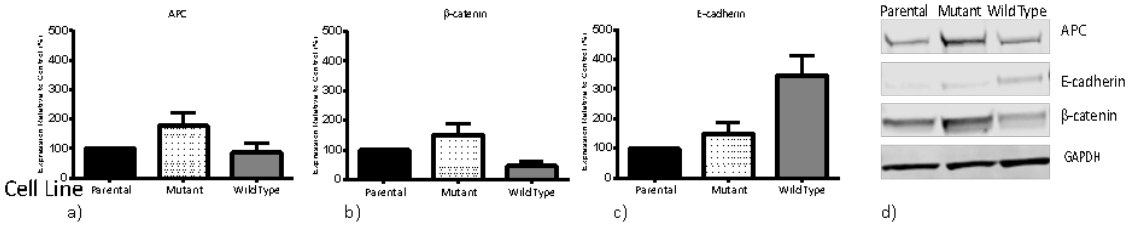



*Figure 3.1: Stabilising β-catenin mutations are associated with different expression levels of APC, β-catenin and E-cadherin. Whole-cell lysates from parental, mutant and wild-type HCT116 cells were subjected to western blot and probed with antibodies against APC, β-catenin and E-cadherin. Total protein levels were normalised to GAPDH. Protein levels of APC (a), β-catenin (b) and E-cadherin (c) are plotted. Data represent the mean and standard deviation of three independent experiments. A representative western blot is shown in (d).*

E-cadherin expression is much higher in the wild-type cells compared with both the parental and mutant cell lines. β-Catenin and APC levels are highest in the mutant cells and lowest in the wild types with the parental cell line showing intermediate expression levels.

Statistical values quoted are based on t-tests unless otherwise stated. Statistical significance level is set at $p < 0.05$). APC levels are approximately two times greater in the mutant compared with parental cells ($p < 0.05$) while the wild-type cells express an APC level equivalent to the parental cells ($p = 0.395$).

The cells also differ in their expression level of β-catenin ($p < 0.05$ in comparison of both mutant and wild-type cells to the parental cell line). Expression of E-cadherin also differs between the parental, mutant and wild-type HCT116 cells ($p < 0.05$ for each comparison). E-cadherin expression in the parental and mutant cells is very low compared with the wild-type cell line.

The low E-cadherin and higher APC in the mutant cells is the opposite pattern to the wild-type cells. This suggests that a point mutation of β-catenin may alter the differential interaction of β-catenin with either APC or E-cadherin. The stabilised β-catenin cannot be degraded. This, in combination with very low levels of E-cadherin may lead one to hypothesise that the mutant cell line may have higher levels of β-catenin mediated transcriptional output.

### 3.2.1.2 Stabilised β-catenin affects the transcription of β-catenin and E-cadherin but not AXIN2.

After defining the total expression levels of β-catenin, APC and E-cadherin, I measured the transcript levels of β-catenin and E-cadherin. In addition, I also measured the transcription of AXIN2, a β-catenin target gene in each of the three cell lines (Figure 3.2).

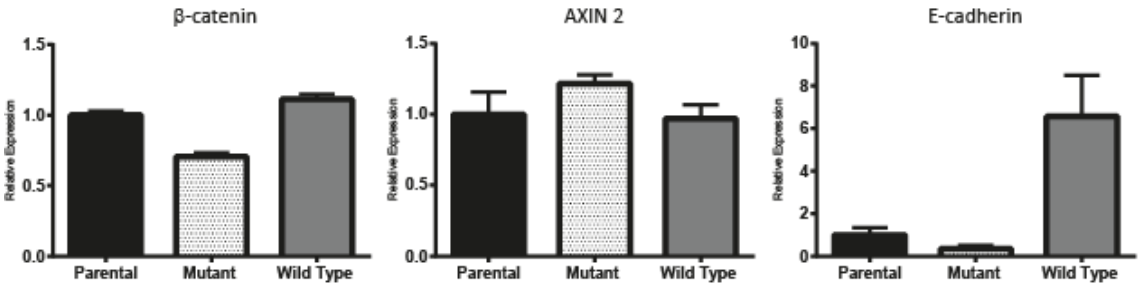



*Figure 3.2: Stabilising β-catenin mutations affect transcription of β-catenin, AXIN2 and E-cadherin in HCT116 cells. Transcript levels of β-catenin (a), AXIN2 (b) and E-cadherin (c) were measured using qPCR in whole-cell lysates of parental, mutant and wild-type HCT116 cells. Plotted values represent the mean and standard deviation of three independent experiments for each condition and levels are plotted relative to the parental cell line.*

E-cadherin transcript levels are significantly higher in the wild-type cell than the parental and mutant cell lines. β-Catenin levels are lower in the mutant compared with both parental and wild-type cells while AXIN2 transcription is similar in all three cell lines.

The elevated E-cadherin protein in the wild type compared with the mutant and parental cells reflects the pattern of transcript expression. Transcript levels of β-catenin in the mutant cell line were significantly lower than the other two cell lines ($p < 0.005$ for both comparisons). This does not reflect the slightly higher level of β-catenin protein in the mutant cells. Perhaps because the stabilised protein is non-degradable, transcription of new β-catenin is subdued to control overall levels.

AXIN2 transcription was not significantly different between the three cell lines studied. This is surprising as the mutant and parental cell lines each express a non-degradable β-catenin protein, and intuitively one may expect this to be reflected by a greater degree of AXIN2 transcription compared with the wild-type cells. In addition, the very low levels of E-cadherin available to bind β-catenin in the parental and mutant cells may have been expected to exacerbate β-catenin transcriptional activity, but it did not.

These findings may indicate that stabilised β-catenin is bound to and sequestered by APC in the mutant and parental cells, although this hypothesis requires further experiments for clarification. These results also indicate that a point mutation causing stabilisation of β-catenin may affect cellular levels of E-cadherin. Figure 3.3 shows a schematic representation of the relationship between β-catenin, APC and e- in the mutant and wild-type cell lines.

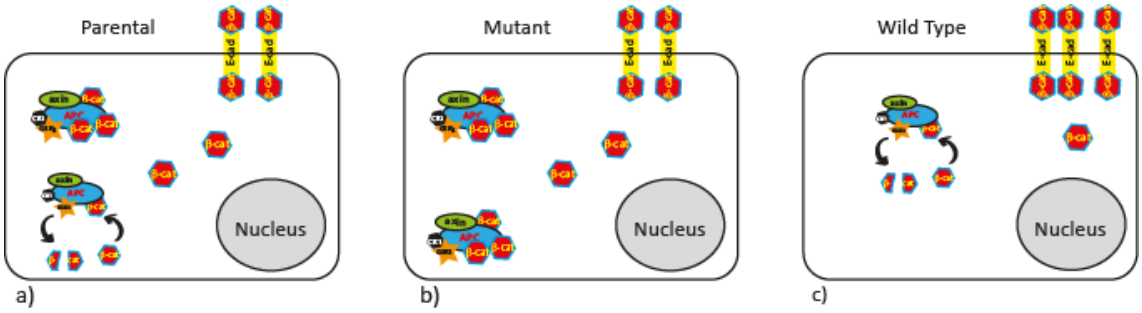



*Figure 3.3: Stabilised β-catenin may be sequestered by APC in parental (a) and mutant (b) HCT116 cells while wild-type β-catenin is degraded by the APC destruction complex in wild-type cells (c). E-cadherin levels are much higher in the wild type (c) compared with the parental and mutant cells (a & b).*

### 3.2.1 Summary of Key Findings:

- Wild-type cells have far more E-cadherin protein and transcript than mutant cells
- Cells expressing only stabilised β-catenin have more APC and less E-cadherin than cells with only wild-type β-catenin.
- AXIN2 transcription is not affected by expression of stabilised β-catenin.

The key differences in protein levels between cells are summarised in Table 3.1.

| Cell Line | APC | β-catenin | E-cadherin |
|---|---|---|---|
| **Parental** | - | - | - |
| **Mutant** | Increased | Increased | Unchanged |
| **Wild Type** | Decreased | Unchanged | Increased |

*Table 3.1: Summary of expression levels of APC, β-catenin and E-cadherin in HCT116 cells. Levels in the mutant and wild-type cells are tabulated relative to the level in the parental cells.*

**3.2.2.1 Expression levels of β-catenin, APC and E-cadherin respond to depletion of each of the three proteins.**

Figure 3.1 revealed that stabilising β-catenin mutations impact on total levels of both APC and E-cadherin. I next wanted to provide further insight into the relationships between these three proteins. I aimed to assess the effect on levels of β-catenin, APC and E-cadherin after depletion of each of these proteins in turn (Figure 3.4).

I also assessed the effect of PTEN depletion on the levels of β-catenin, APC and E-cadherin. The rationale for these experiments is as follows. APC is commonly mutated in colorectal cancer at an early stage in tumour development. The cancer cell however, accumulates further mutations as it progresses from a benign, to malignant and metastatic lesion. PTEN is also commonly mutated in colorectal cancer, implicated in both the conventional and serrated pathways of carcinogenesis. The combination of mutations in a tumour may influence tumour behaviour and impact on outcome. I wanted to assess if loss of PTEN could alter the relationships between β-catenin, APC and E-cadherin. This may indicate potential crosstalk between pathways affected by loss of APC or PTEN. Tumours with mutations in APC or PTEN in isolation may behave differently from tumours with these mutations combined. I aimed to assess changes at a molecular level that may explain differences in tumour features.

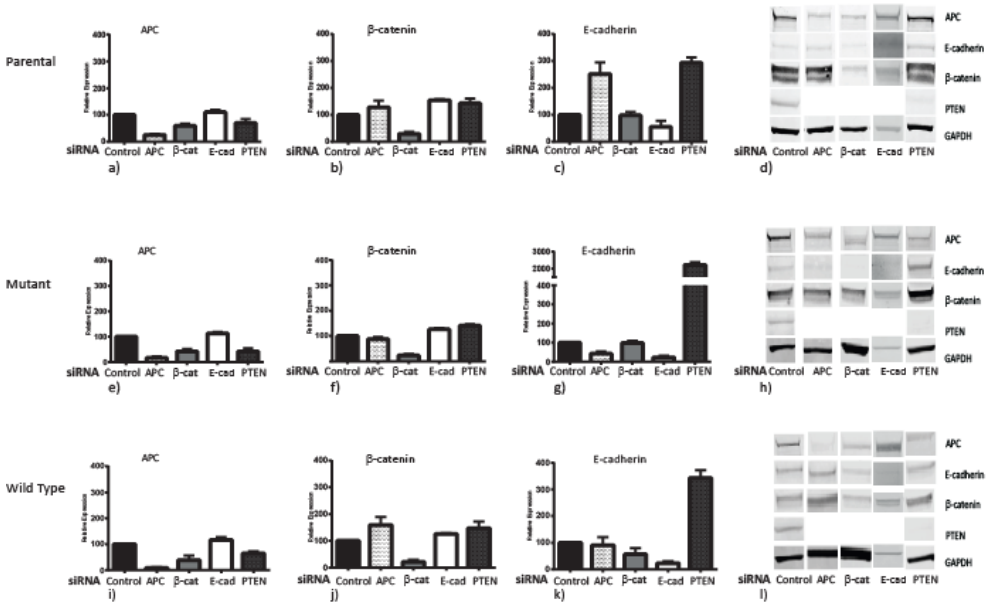



*Figure 3.4: siRNA targeted depletion of APC, β-catenin, E-cadherin or PTEN can influence expression levels of APC, β-catenin and E-cadherin. APC, β-catenin, E-cadherin or PTEN were depleted in Parental (top panel), Mutant (middle panel) and Wild-Type (bottom panel) HCT116 cells. Whole-cell lysates were then subjected to western blot and probed with antibodies against APC, E-cadherin, β-catenin and GAPDH. Protein levels were measured relative GAPDH. Plotted values represent the mean and standard deviation of three independent experiments are presented relative to levels under control conditions. Levels of APC are plotted in (a, e & i), β-catenin in (b, f & j) and E-cadherin in (c, g & k). Representative western blots are shown in (d, h & l).*

The most striking findings from these experiments are that depletion of PTEN causes an increase in E-cadherin in all cell lines studied. APC depletion only causes modest increases in β-catenin protein levels in the parental and wild-type cells with no change in the mutant cell line. Surprisingly APC levels fall following depletion of β-catenin in all cell lines.

E-cadherin levels rose significantly in all cell lines following knockdown of PTEN (c, g & k). The magnitude of the rise here should be interpreted with caution in the parental and mutant cells as they both had very low baseline levels so a degree of measurement artefact is possible. Modest increases in β-catenin were also detected following PTEN depletion so perhaps some of the increase in E-cadherin can be explained by stabilisation of the protein. The β-catenin increase following PTEN loss could potentially relate to activation of the Akt pathway and

subsequent inhibition of GSK3β, which may negatively impact on the function of the APC destruction complex (Maurer *et al.* 2014). PTEN was only blotted under control and PTEN knock down conditions. This was done to show that the PTEN knock down worked. It was not relevant for inclusion under the other conditions studied.

Despite siRNA treatment causing APC levels to fall to 5-10% of control levels, APC depletion caused only a modest rise in β-catenin in the parental and wild-type cells and no change in the mutant cells (b, f & j). No rise is expected following loss in the mutant cells as the stabilised β-catenin is degradation resistant and the changes in the other two cell lines are in keeping with a loss of β-catenin degradation.

Surprisingly APC levels also fell following β-catenin depletion in all cell lines (a, e & i). The mechanisms for this are unclear but may represent a feedback loop. If less β-catenin is available for APC to degrade or sequester, perhaps less APC is required to regulate β-catenin, potentially explaining the reduction in APC protein level.

#### 3.2.2.2 Transcription levels of β-catenin, AXIN2 and E-cadherin respond to depletion of β-catenin, E-cadherin APC or PTEN

I next aimed to establish if the measured changes in protein levels of β-catenin and E-cadherin were reflected by changes in transcript level. In addition, I measured the transcriptional output of β-catenin by quantifying transcription of AXIN2 (Figure 3.5).

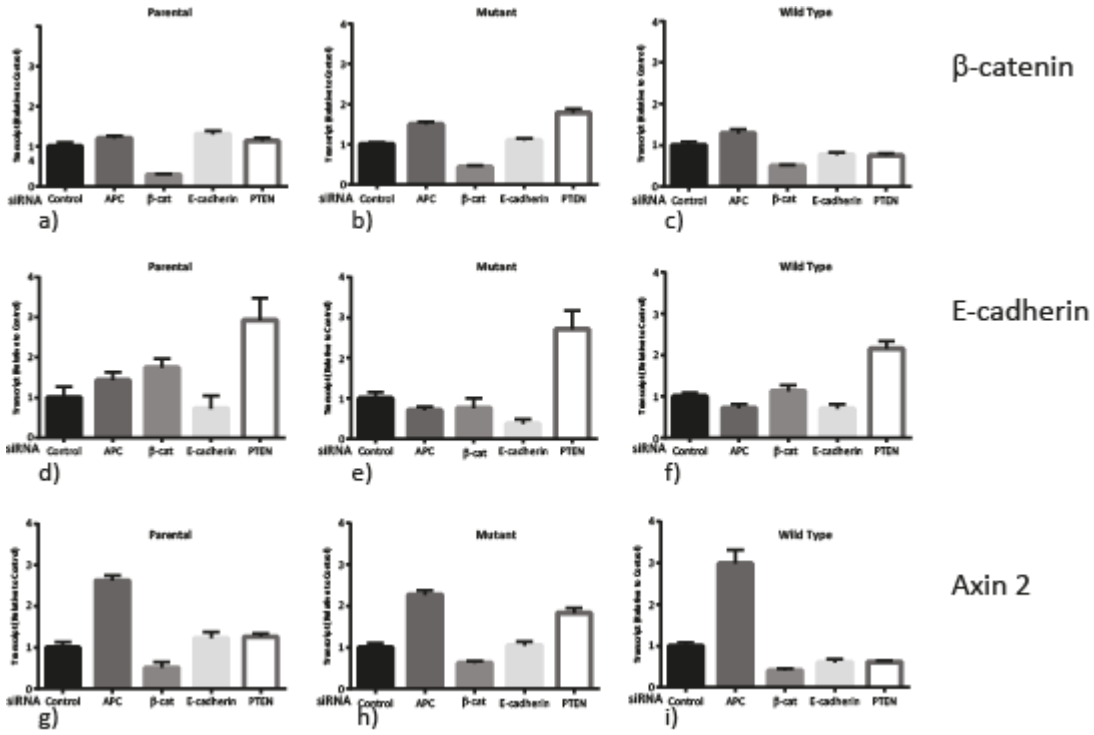



*Figure 3.5: Transcript levels of β-catenin, E-cadherin and AXIN2 are responsive to depletion of APC, β-catenin, E-cadherin and PTEN. Transcript levels of β-catenin (a–c)), E-cadherin (d–f) and AXIN2 (g–i) were measured using qPCR in whole-cell lysates of parental, mutant and wild-type HCT116 cells. Plotted values represent the mean and standard deviation of three independent experiments for each condition and levels are plotted relative to the parental cell line.*

E-cadherin transcript level increases significantly following PTEN depletion in all cell lines. AXIN2 transcription increases significantly in all cell lines following APC depletion. In all cell lines, the transcript level of β-catenin increases following APC depletion.

Transcriptional increases in β-catenin following APC depletion are surprising. The accepted mechanism of β-catenin increase after APC loss is attributed to a loss of degradative function. These data suggest that *de novo* transcription may also contribute. This increase in transcript follows the increase in β-catenin levels observed in the parental and wild-type cells. The mutant cell line showed no increase in protein following APC depletion, although the transcript level is increased slightly.

The increase in E-cadherin transcript reflects the increased E-cadherin protein level in all cell lines following depletion of PTEN. This indicates that the increase in E-cadherin protein level is at least in part due to new transcription as opposed to stabilisation of the E-cadherin protein. The mechanisms responsible for this increase are unclear and require further experiments for clarification.

AXIN2 transcript increases following APC depletion in all cell lines. This may be stimulated by a slight increase in β-catenin under these conditions in the parental and wild-type cells. The mutant cells had no increase in β-catenin protein level following APC loss so these data may support the hypothesis that stabilised β-catenin is sequestered by APC. Release of this sequestered pool could contribute to AXIN2 transcriptional increases in the parental and mutant cell line.

Changes in protein and transcriptional levels of the proteins under study are summarised in Table 3.2.

| Parental Cells | Control | APC KD | | β-cat KD | | E-cad KD | | PTEN KD | |
|---|---|---|---|---|---|---|---|---|---|
| | – | Protein | Transcript | Protein | Transcript | Protein | Transcript | Protein | Transcript |
| APC | – | Down | – | Down | – | Up | – | Down | – |
| β-catenin | – | Up | Up | Down | No change | Up | No change | Up | No change |
| E-cadherin | – | Up | No change | No Change | Up | Down | Down | Up | Up |
| AXIN2 | – | – | Up | – | Down | – | Up | – | Up |

| Mutant Cells | Control | APC KD | | β-cat KD | | E-cad KD | | PTEN KD | |
|---|---|---|---|---|---|---|---|---|---|
| | – | Protein | Transcript | Protein | Transcript | Protein | Transcript | Protein | Transcript |
| APC | – | Down | - | Down | – | Up | – | Up | – |
| β-catenin | – | No Change | Up | Down | No change | Up | No change | Up | Up |
| E-cadherin | – | Up | No change | No Change | No change | Down | Down | Up | Up |
| AXIN2 | – | – | Up | - | Down | – | Up | – | Up |

| Wild-Type Cells | Control | APC KD | | β-cat KD | | E-cad KD | | PTEN KD | |
|---|---|---|---|---|---|---|---|---|---|
| | – | Protein | Transcript | Protein | Transcript | Protein | Transcript | Protein | Transcript |
| APC | – | Down | – | Down | – | Up | – | Up | – |
| β-catenin | – | Up | Up | Down | No change | Up | No change | Up | No change |
| E-cadherin | – | No Change | No change | No Change | No change | Down | Down | Up | Up |
| AXIN2 | – | – | Up | – | Down | – | No change | – | No Change |

*Table 3.2: Summary of directional changes relative to the control in levels of protein (blue) and transcript (green) levels of APC, β-catenin, E-cadherin and AXIN2 in response to depletion of APC, β-catenin, E-cadherin or PTEN are presented. Red text indicates results of interest.*

### 3.2.2 Summary of Key Findings:

- APC protein levels decrease following β-catenin depletion in all cell lines studied.
- APC depletion caused a modest increase in β-catenin protein level in cells with wild-type β-catenin but caused no change in those with only mutant β-catenin; however, β-catenin transcript level increased in all cell lines.
- APC depletion caused a significant increase in AXIN2 transcription in all cell lines.
- E-cadherin protein and transcript levels rose in response to PTEN depletion in all cell lines.

### 3.2.3 Depletion of APC and E-cadherin causes a redistribution of β-catenin from the plasma membrane to the cytoplasm

I next aimed to measure where in the cell β-catenin was located, both under control conditions and following depletion of APC, β-catenin, E-cadherin and PTEN. I first studied the cells under control conditions using immunofluorescence microscopy. This allowed visual comparison of the overall levels of β-catenin and E-cadherin. It also generated a picture of where in the cell these proteins were localised (Figure 3.6).

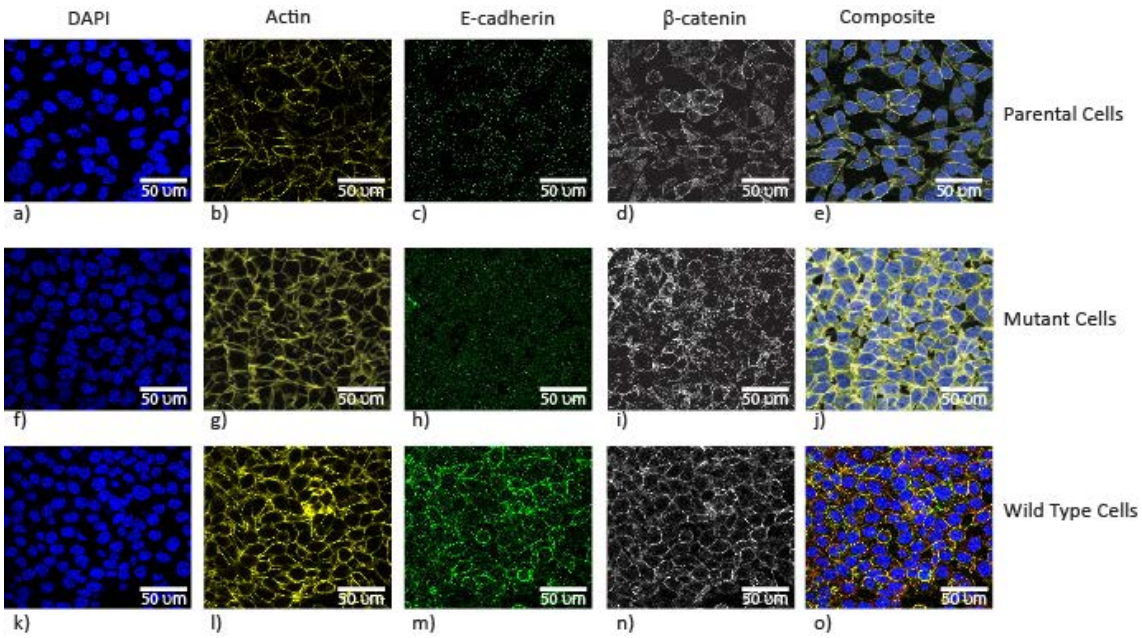



*Figure 3.6: Immunoflourescent microscopy shows a predominantly membrane-associated β-catenin in all cell lines studied. Parental (a–e), mutant (f–j) and wild type (k–o) HCT116 cells were fixed and stained with DAPI (a, f & k) and phalloidin (b, g & l) to identify the cell nucleus and cell periphery. Antibodies against E-cadherin (c, h & m) and β-catenin (d, I & n) were used to indicate their location. Composite images (e, j & o) were generated by combinations of DAPI, phalloidin, E-cadherin and β-catenin images. Scale bar represents 50 μm.*

The images show a predominant membrane localisation for β-catenin in all cell lines. Very low levels of E-cadherin were detected in the parental and mutant (c & h) cells with slightly more in the wild-type (m) cells. This supports biochemical data in Figure 3.1.

I next aimed to determine the localisation of β-catenin biochemically by fractionating cells into cytoplasmic, membrane, nuclear and cytoskeletal pools. In addition, I established if the localisation of β-catenin could be changed by depletion of APC, β-catenin, E-cadherin or PTEN (Figure 3.7). Prior to cell fractionation the cells were treated with siRNa to deplete target proteins. This was carried out in exactly the same manner as the experiment above that assessed total protein levels following depletion of target proteins

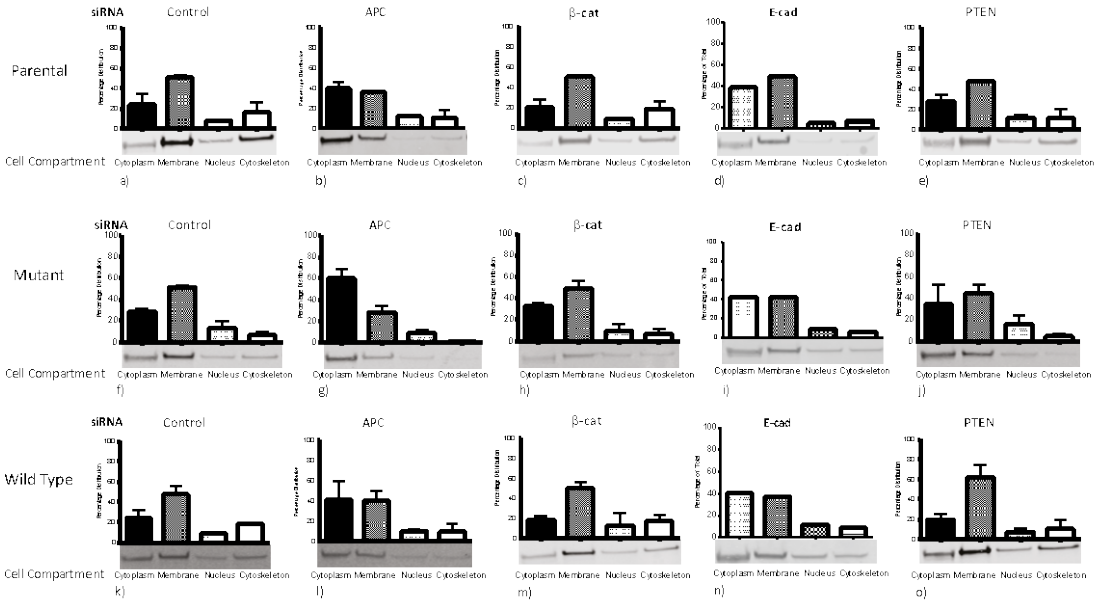



*Figure 3.7: Cellular location of β-catenin is affected by depletion of APC (b, g & l) and E-cadherin (d, I & n) in all cell lines studied. Cells were fractionated into cytoplasmic, membrane, nuclear and cytoskeletal compartments. Lysates were then subjected to western blot and probed with antibody against β-catenin. Proportional distribution between cell compartments was then plotted. Data represent the mean and standard deviation of three independent experiments except E-cadherin depletion, which represents a single experiment. Results obtained from the parental cell line are shown (top row), mutant (middle row) and wild type (bottom row).*

Under control conditions β-catenin is located mainly at the cell membrane and all cell lines show a similar distribution throughout the cell compartments. Depletion of APC caused a redistribution of β-catenin from the cell membrane to the cytoplasm in all cell lines studied. E-cadherin knockdown led to a similar cytoplasmic redistribution.

The distribution of β-catenin under control conditions (a, f & k) is reasonably consistent in all three cells. Approximately 50–60% of total β-catenin resides in the membrane compartment of the cells with 20–30% in the cytoplasm. The cytoskeletal compartment contains approximately 20% of β-catenin, while nuclear β-catenin accounts for 10%. The exception to this is the mutant cells, in which the cytoskeletal and nuclear pools make up 30% and 20%, respectively.

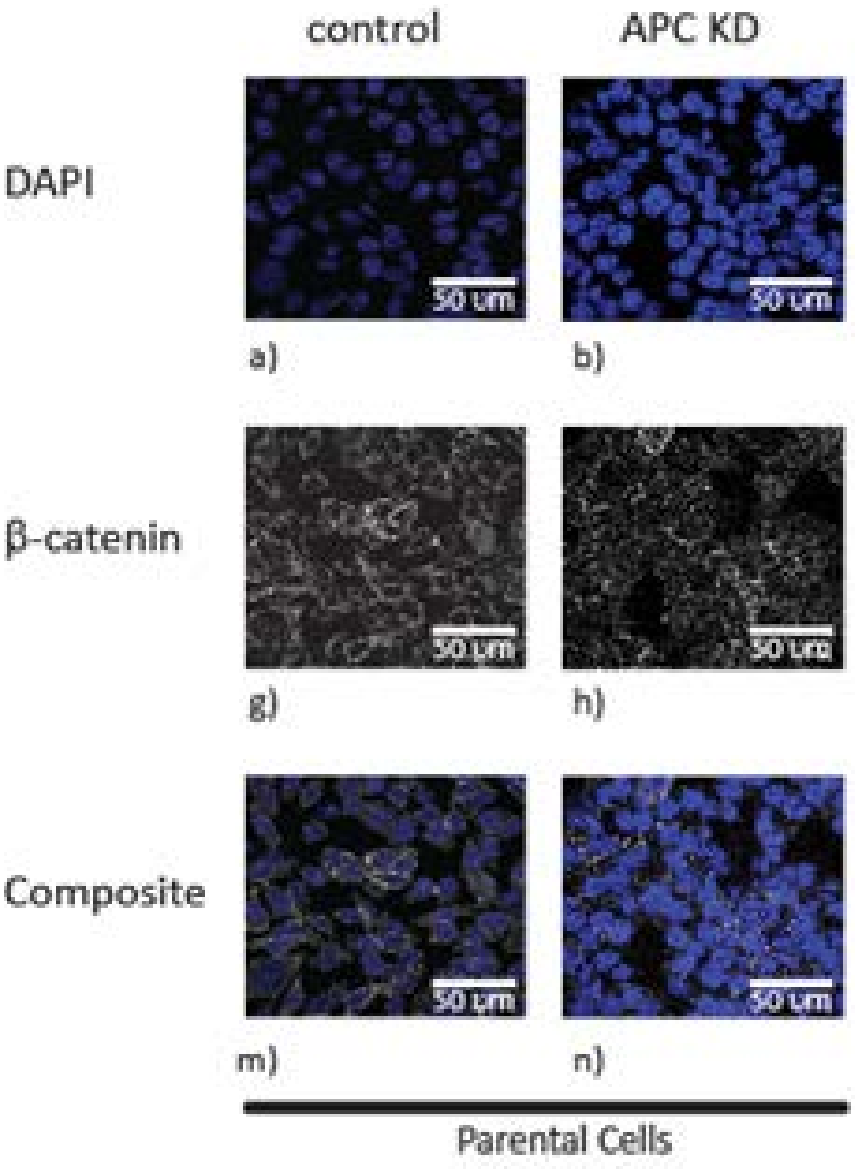
Based on these figures, the greatest proportion of β-catenin appears to be associated with E-cadherin at the cell membrane under control conditions. It is worth recollecting that the parental

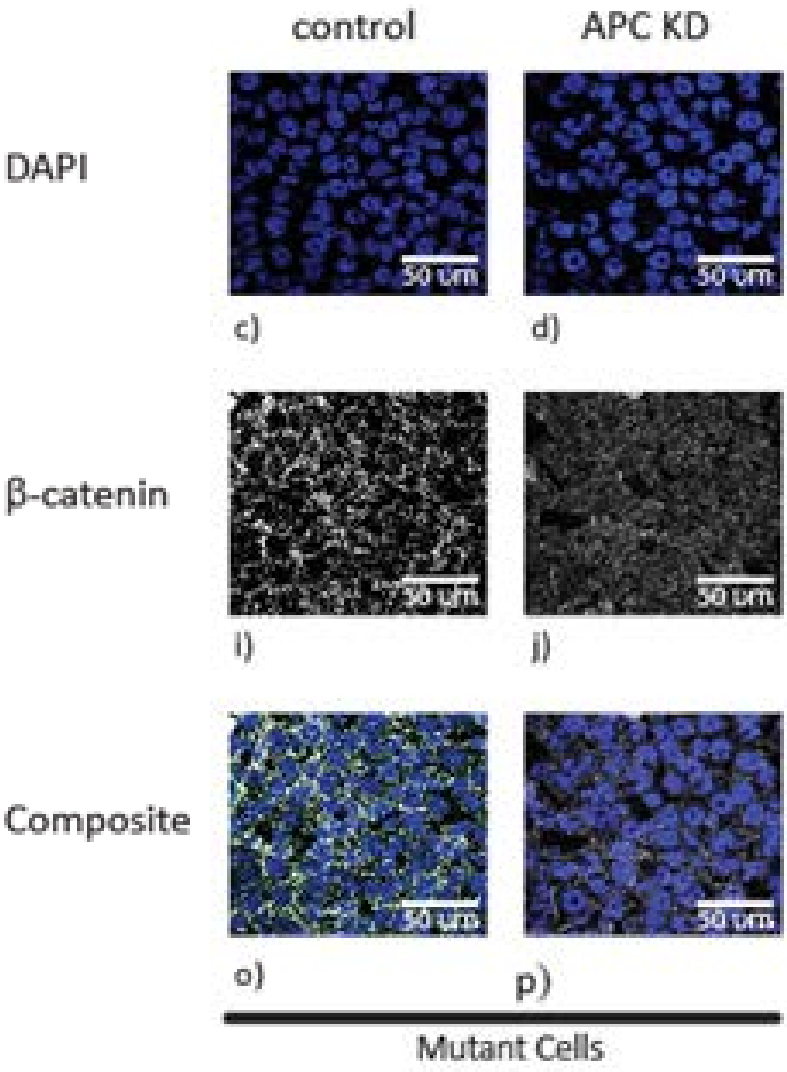
and mutant cell lines have low levels of E-cadherin, suggesting that the β-catenin is recruited to the membrane region by alternative binding partners, other than E-cadherin.

APC knockdown led to a marked redistribution of β-catenin from the membrane to the cytoplasm in all cell lines. The redistribution in the parental and mutant cells was statistically significant but did not reach significance in the wild-type cells (b, j & l). Loss of APC in the mutant cells does not affect total β-catenin levels as degradation is not altered. The redistribution here may therefore be due to release of sequestered β-catenin from APC. This implies that the interaction between APC and β-catenin occurs in the membrane region of the cell.

E-cadherin depletion caused a cytoplasmic redistribution of β-catenin from the membrane in all cell lines (d, I & n). This resulted in an approximate matching of cytoplasmic and membrane β-catenin proportions in all cell lines. The redistribution may be expected, as loss of E-cadherin leads to dissociation of the adherens junctions and release of β-catenin. This matched very closely the proportional redistribution seen after APC depletion. The exception to this was the mutant cell line, where the cytoplasmic pool was greater after APC KD than E-cadherin depletion. This may reflect the finding that the mutant cell line has very low levels of E-cadherin.

In support of the cell fractionation data, I also obtained Immunoflourescent images of all three cell lines following APC depletion to compare visually the localisation of β-catenin to control conditions (Figure 3.8).


control
APC KD
DAPI
50 um
a)
b)
β-catenin
g)
h)
Composite
m)
n)
Parental Cells


control
APC KD
DAPI
β-catenin
Composite
50 um
c)
d)
i)
j)
o)
p)
Mutant Cells

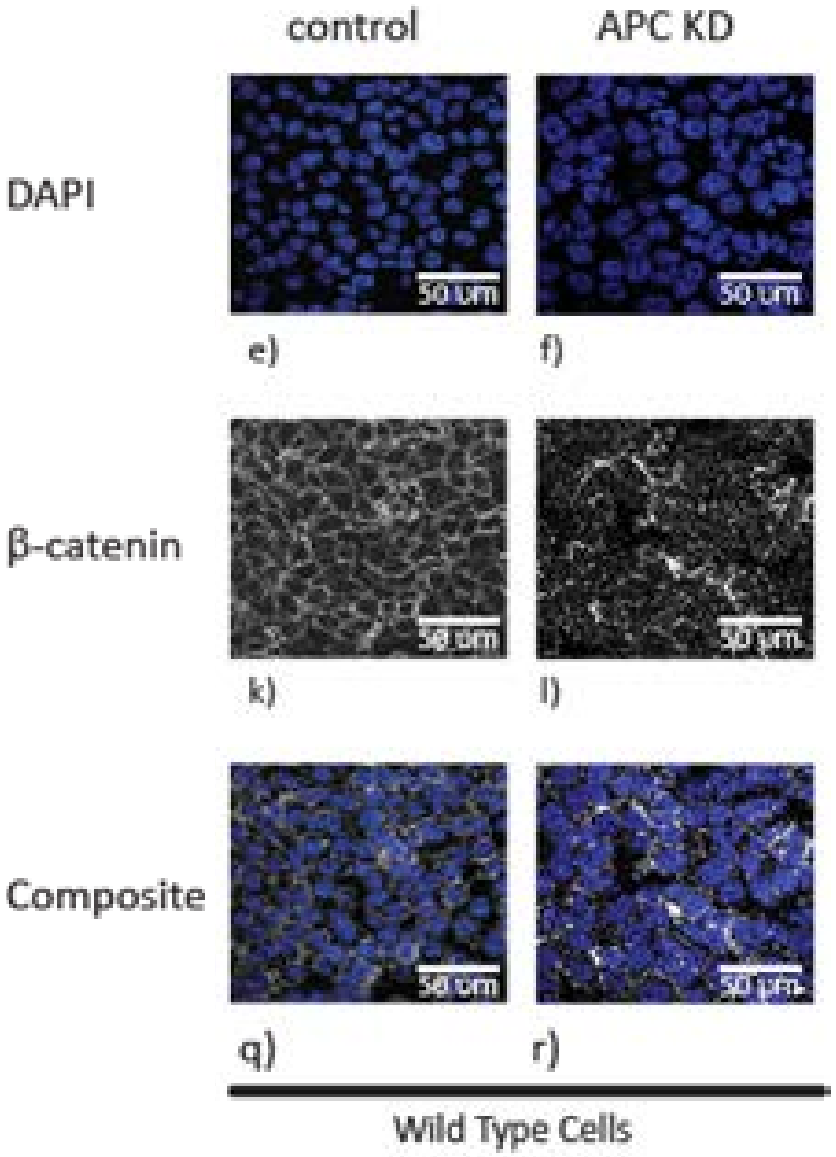



*Figure 3.8: APC depletion causes a redistribution of β-catenin from the plasma membrane to the cytoplasm in all cell lines. HCT116 cells were fixed and stained with DAPI (a–f) and probed with β-catenin antibody (g–l). Composite images (m–r) were generated by combination of the DAPI and β-catenin images. Scale bar represents 50 μm.*

In all cells lines, APC depletion caused a reduction in the intensity of the β-catenin signal at the cell periphery. This was accompanied by an increase of β-catenin signal in the cytoplasm, in support of the cell fractionation data.

I have suggested that stabilised β-catenin may be sequestered by APC. It is possible that if much of the cell's APC is occupied by sequestered β-catenin then it will be unavailable to perform

alternative functions. To investigate this, I measured the location of APC using immunofluorescent microscopy under control conditions and compared this with cells in which β-catenin had been depleted (Figure 3.9).

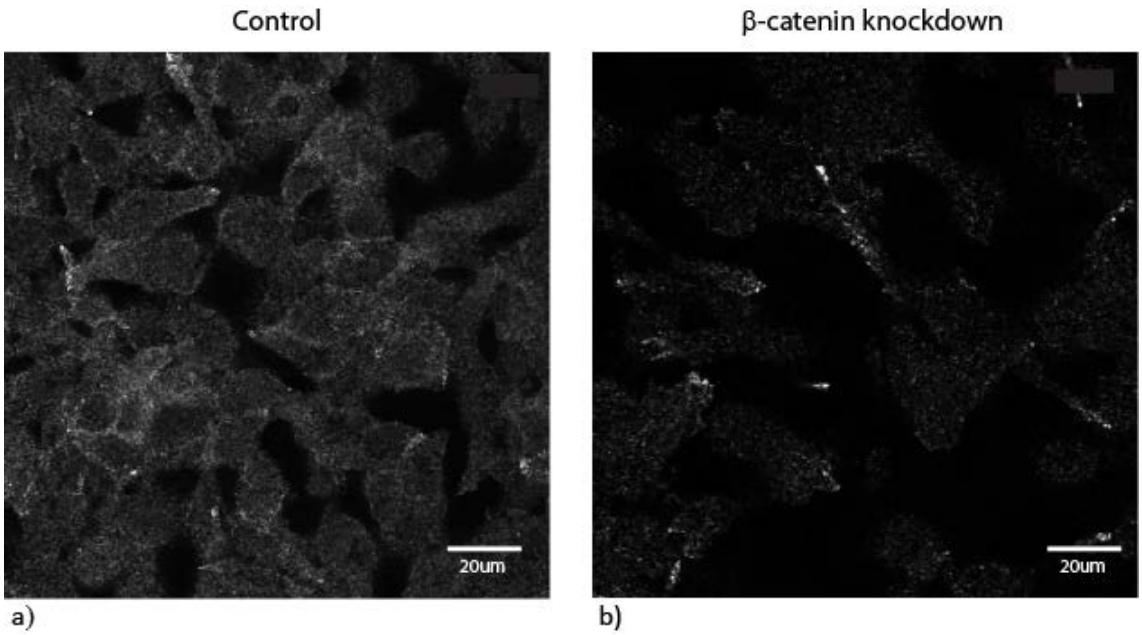



*Figure 3.9: Depletion of β-catenin in HCT116 cells causes APC to localise in distinct clusters characteristic of localisation at microtubule ends. Mutant HCT116 cells were probed with antibody against APC under control (a) and β-catenin depleted (b) conditions. Scale bar represents 20 μm.*

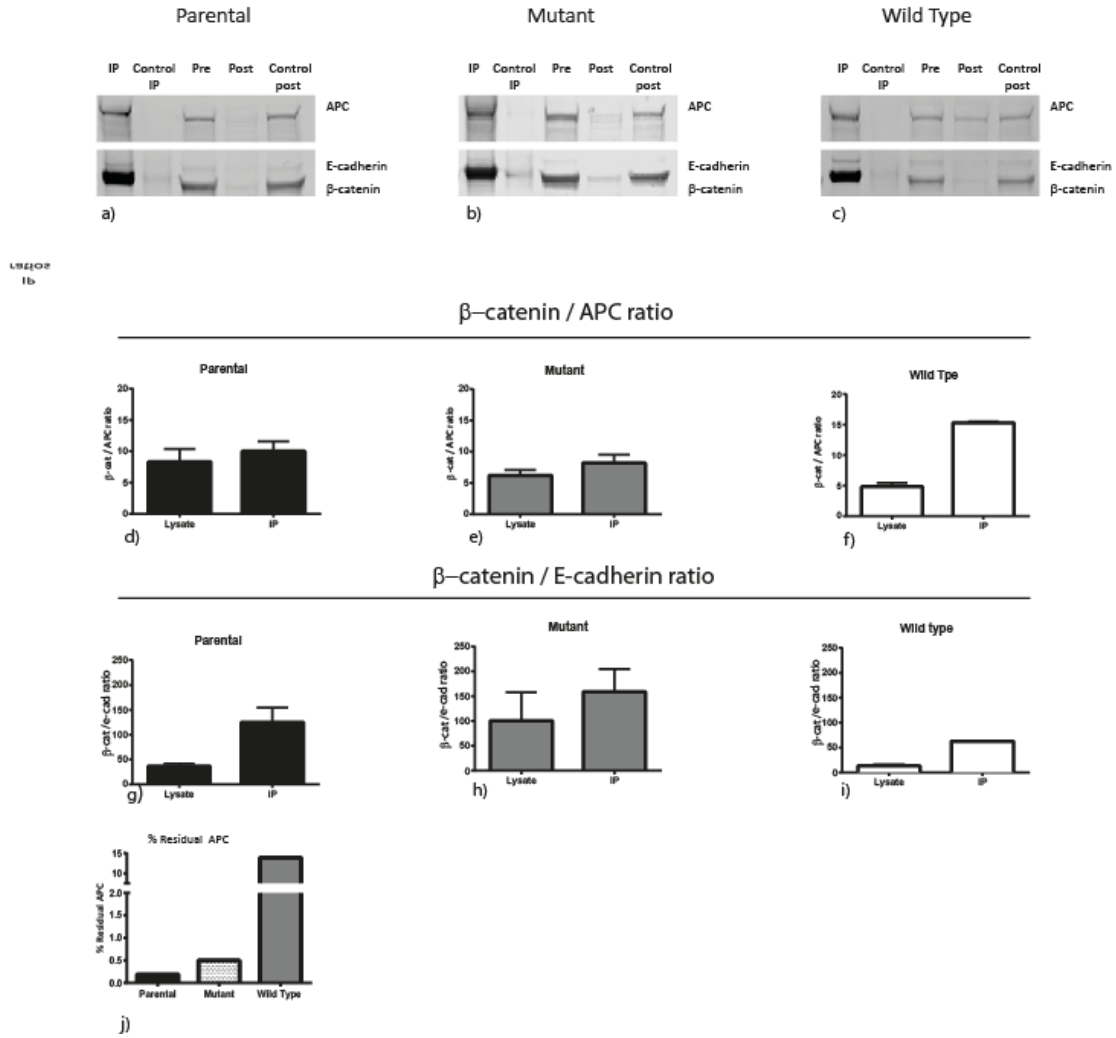
Following β-catenin depletion, APC localises in clusters in keeping with localisation at microtubule ends. APC has been reported in this location previously (Nathke *et al.* 1996). This result highlights how the contribution of APC to one role in the cell may be affected by another. In the mutant cell line examined, APC sequesters β-catenin. Depletion of β-catenin results in a dramatic shift of APC to microtubule ends. This indicates that if APC is relieved of a specific role (in this case sequestration of β-catenin) it may then be available to participate in alternative functions.

### 3.2.3 Summary of Key Findings:

- The interactions β-catenin/E-cadherin and β-catenin/APC occur in the membrane region of the cell.
- APC depletion causes a marked redistribution of β-catenin from the membrane to the cytoplasm.
- E-cadherin depletion causes a marked redistribution of β-catenin from the membrane to the cytoplasm.
- Depletion of β-catenin results in the accumulation of APC at microtubule ends.

### 3.2.3 Stabilised β-catenin binds more tightly to APC and less tightly to E-cadherin

I have suggested that a point mutation causing stabilisation of β-catenin results in sequestration by APC. The binding sites for APC and E-cadherin on β-catenin overlap, so interaction with each of these two partners is mutually exclusive (Valenta *et al.* 2012). I next aimed to determine if more stabilised β-catenin could be found in association with APC than wild-type β-catenin. In addition, I measured the level of interaction between both mutant and wild-type β-catenin with E-cadherin. A β-catenin co-immunoprecipitation (Co-IP) was used to investigate these interactions (Figure 3.10).




| Parental | β-cat/APC ratio (IP) | β-cat /E-cad ratio (IP) | β-cat/APC ratio (lysate) | β-cat /E-cad (lysate) |
|---|---|---|---|---|
| Mean | 10 | 125.2 | 9.4 | 40 |
| SD | 1.6 | 29.3 | 0.9 | 9 |

| Mutant | β-cat/APC ratio (IP) | β-cat /E-cad ratio (IP) | β-cat/APC ratio (lysate) | β-cat /E-cad (lysate) |
|---|---|---|---|---|
| Mean | 8.2 | 159 | 6.3 | 94 |
| SD | 1.3 | 46 | 1.4 | 74 |

| Wild Type | β-cat /APC ratio (IP) | β-cat/E-cad ratio (IP) | β-cat/APC ratio (lysate) | β-cat /E-cad (lysate) |
|---|---|---|---|---|
| Mean | 15.3 | 62.6 | 4.7 | 12 |
| SD | 0.2 | 1 | 1.2 | 2 |

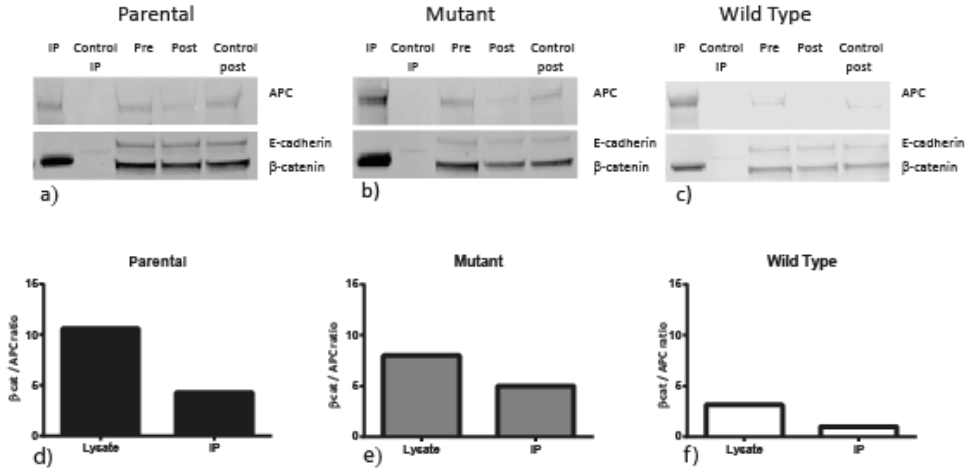
*Figure 3.10: Stabilising β-catenin mutations increases its interaction with APC. β-Catenin was co-immunoprecipitated and western blot against APC, β-catenin and E-cadherin performed on the IP and lysates. Representative blots are shown in parental (a), mutant (b) and wild-type cells (c). The ratio of β-catenin/APC in the IP and lysates for each cell line are plotted in (d–f) and the β-catenin/E-cadherin ratio is plotted in (g–i). Plots represent the mean and standard deviation of three independent experiments. Residual APC in the post-IP lysate is plotted in (j). Tabulated data are shown below the graphs.*

Stabilised β-catenin interacts more with APC than does wild-type β-catenin. More E-cadherin is found in association with wild-type β-catenin than with stabilised β-catenin.

Stabilised β-catenin in the parental and mutant cells has a greater interaction with APC than the β-catenin in the wild-type cells. The β-catenin/APC ratios in the IP of parental and mutant cells are similar at 10 and 8.2, respectively, which are similar to the ratios in the lysate of these cells (9.4 and 6.3). These are both lower than the ratio of 15.3 in the wild-type cells' IP. The β-catenin/APC ratio in the wild-type lysate is also lower at 4.7 than the ratio in the wild-type IP (15.3). Together, these results indicate that more APC is associated with β-catenin in the parental and mutant cells compared with the wild-type cells. Considering that the stabilised β-catenin cannot be degraded by APC, these data indicate that the relationship between APC and stabilised β-catenin serves a sequestration function whereby β-catenin can be bound by APC but not degraded. A greater degree of interaction between stabilised β-catenin and APC is reflected by the higher level of residual APC left in the post-IP lysate of the wild-type cells. This indicates that in these cells not all APC is saturated with β-catenin, whereas in cells with stabilised β-catenin it largely is.

The interaction between wild-type β-catenin and E-cadherin is greater than that between stabilised β-catenin and E-cadherin reflected by the findings of the IP. The parental and mutant cells express very low levels of E-cadherin so the low levels of interaction between β-catenin and E-cadherin could be a reflection of the fact that there is little E-cadherin available to which to bind. An alternative explanation is that much of the stabilised β-catenin is associated with APC, so is therefore unavailable to bind to E-cadherin. Another hypothesis is that the stabilised β-catenin may be unable to bind efficiently to E-cadherin, resulting in low levels of interaction and a low expression of E-cadherin.

To investigate these relationships further, I immunoprecipitated APC and measured the amount of β-catenin that was recovered in the different cell types (Figure 3.11).




| *Parental* | *Lysate* | *IP* |
|---|---|---|
| *β-cat/APC ratio* | *10.6* | *4.3* |

| *Mutant* | *Lysate* | *IP* |
|---|---|---|
| *β-cat/APC ratio* | *8* | *5* |

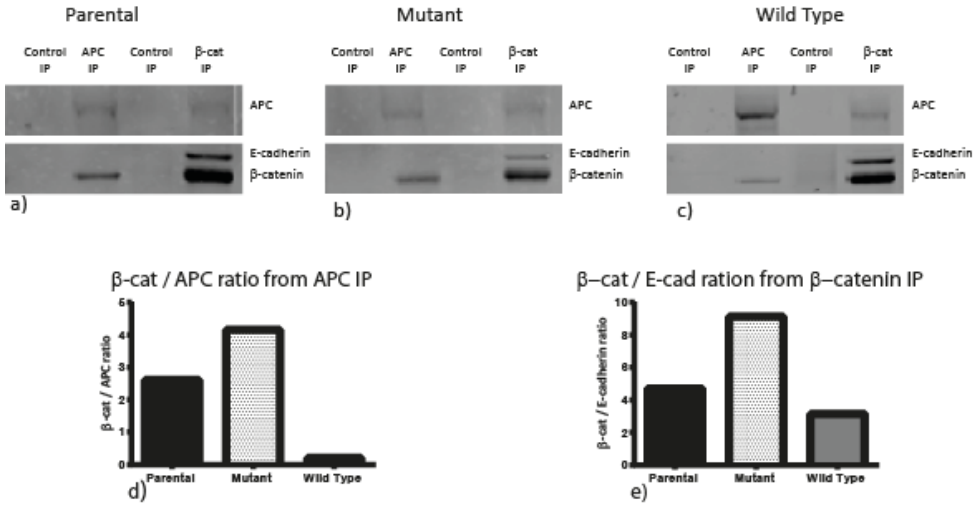
| *Wild Type* | *Lysate* | *IP* |
|---|---|---|
| *β-cat/APC ratio* | *3.15* | *1* |

*Figure 3.11: Stabilising β-catenin mutations increases its interaction with APC. APC was co-immunoprecipitated with western blot against APC, β-catenin and E-cadherin. Representative western blots for the IP and lysate in parental (a), mutant (b) and wild-type cells (c). β-Catenin/APC ratios in the lysate and IP are plotted for each cell line in (d–f) with tabulated data shown below the graphs.*

There is more β-catenin bound to APC in the mutant cells compared with wild type, with the parental cells showing an intermediate result that is more aligned with the mutant cells. These results support those obtained in the β-catenin IP and the hypothesis that stabilised β-catenin is sequestered by APC. Evidence of the mutual exclusivity of the binding of β-catenin to either APC or E-cadherin was also confirmed here. No E-cadherin was present in the IP, although it was detected in the pre- and post-IP lysate.

Next, I aimed to see if I could recover different pools of β-catenin bound to either APC or E-cadherin. I first precipitated APC Co-IP, and then precipitated β-catenin from the post-IP lysates. This allowed me to determine if any, or all of the β-catenin that remained after removing APC

was found bound to E-cadherin. In addition, I wanted to determine the relationship between the relative amounts of β-catenin bound to APC and E-cadherin (Figure 3.12).




| Cell | Parental | Mutant | Wild Type |
|---|---|---|---|
| **β-cat/APC ratio** | 2.61 | 4.15 | 0.2 |

| Cell | Parental | Mutant | Wild Type |
|---|---|---|---|
| **β-cat /E-cad ratio** | 4.71 | 9.13 | 3.15 |

*Figure 3.12: Recovering APC-associated β-catenin reveals differential binding of β-catenin to E-cadherin in HCT116 cells. APC was co-immunoprecipitated then β-catenin was co-immunoprecipitated on the post-IP lysate cleared of APC-bound β-catenin. Western blots for APC, E-cadherin and β-catenin in parental (a), mutant (b) and wild-type cells (c) are shown. The β-catenin/APC ratio from the APC IP is plotted in (d) and the β-catenin/E-cadherin ratio from the β-catenin IP is plotted in (e). Ratio data are tabulated below.*

Relatively less β-catenin is bound to APC in the wild type compared with mutant cell lines, whereas relatively more β-catenin is associated with E-cadherin in the wild type compared with the mutant cells. These results add further support to conclusions from the comparative single immunoprecipitations described above.

These experiments revealed that stabilising β-catenin mutations shifts the distribution of β-catenin to an APC-associated pool. This increased interaction with APC comes at the detriment of interaction with E-cadherin. This is especially apparent in the parental and mutant cell lines. The converse is true in the wild-type cells, where proportionally less β-catenin is found in

association with APC, but more is identified bound to E-cadherin. These interactions are schematically represented for a mutant and a wild-type cell (Figure 3.13).

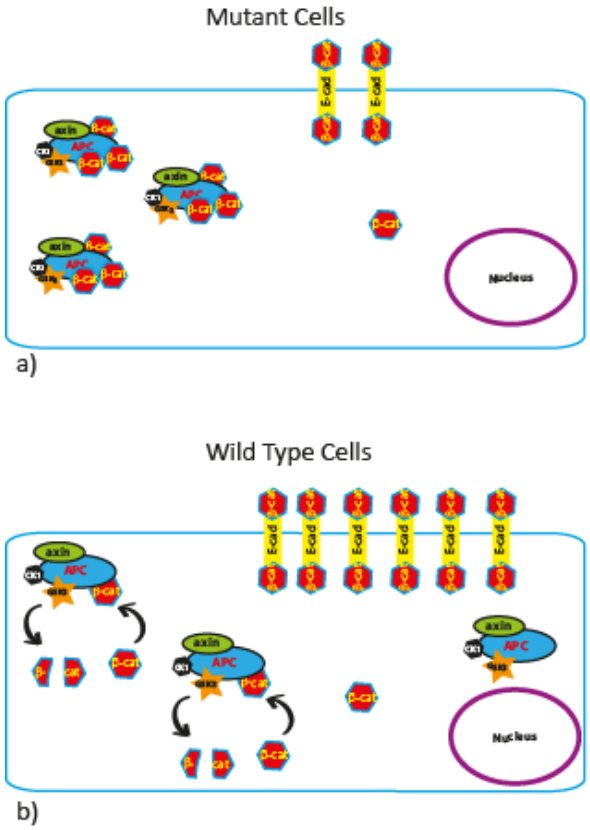



*Figure 3.13: β-Catenin is sequestered by APC in the mutant cell line (a) and little is associated with E-cadherin, which is expressed at a low level. The interaction of β-catenin with APC in the wild-type cells (b) is transient and serves a degradative function. Higher levels of E-cadherin are expressed in the wild-type cells and a higher proportion of β-catenin is identified in conjunction with E-cadherin.*

### 3.2.4 Summary of Key Findings:

- Relatively more stabilised β-catenin is found in association with APC compared with wild-type β-catenin
- Relatively more wild-type β-catenin is identified bound to E-cadherin than stabilised β-catenin

### 3.2.5 Overexpression of β-catenin in HCT116 cells

I next wanted to assess what impact overexpression of either a mutant non-degradable or wild-type β-catenin allele had on both mutant and wild-type HCT116 cells.

The aim of these experiments was to measure if the level of APC or E-cadherin proteins was responsive to over-expression of β-catenin. In addition, I wanted to determine where in the cell exogenous β-catenin localised and if this changed the localisation of endogenous β-catenin. I also measured the expression level of Snail because it has been implicated in the down-regulation of E-cadherin in the process of EMT (Barrallo-Gimeno and Nieto 2005). This transcriptional repression of E-cadherin by Snail can be caused by elevated β-catenin (Zhou *et al.* 2004, Yook *et al.* 2006). I first quantified the levels of APC, β-catenin and E-cadherin following transfection of either a mutant or wild-type β-catenin allele (Figure 3.14).

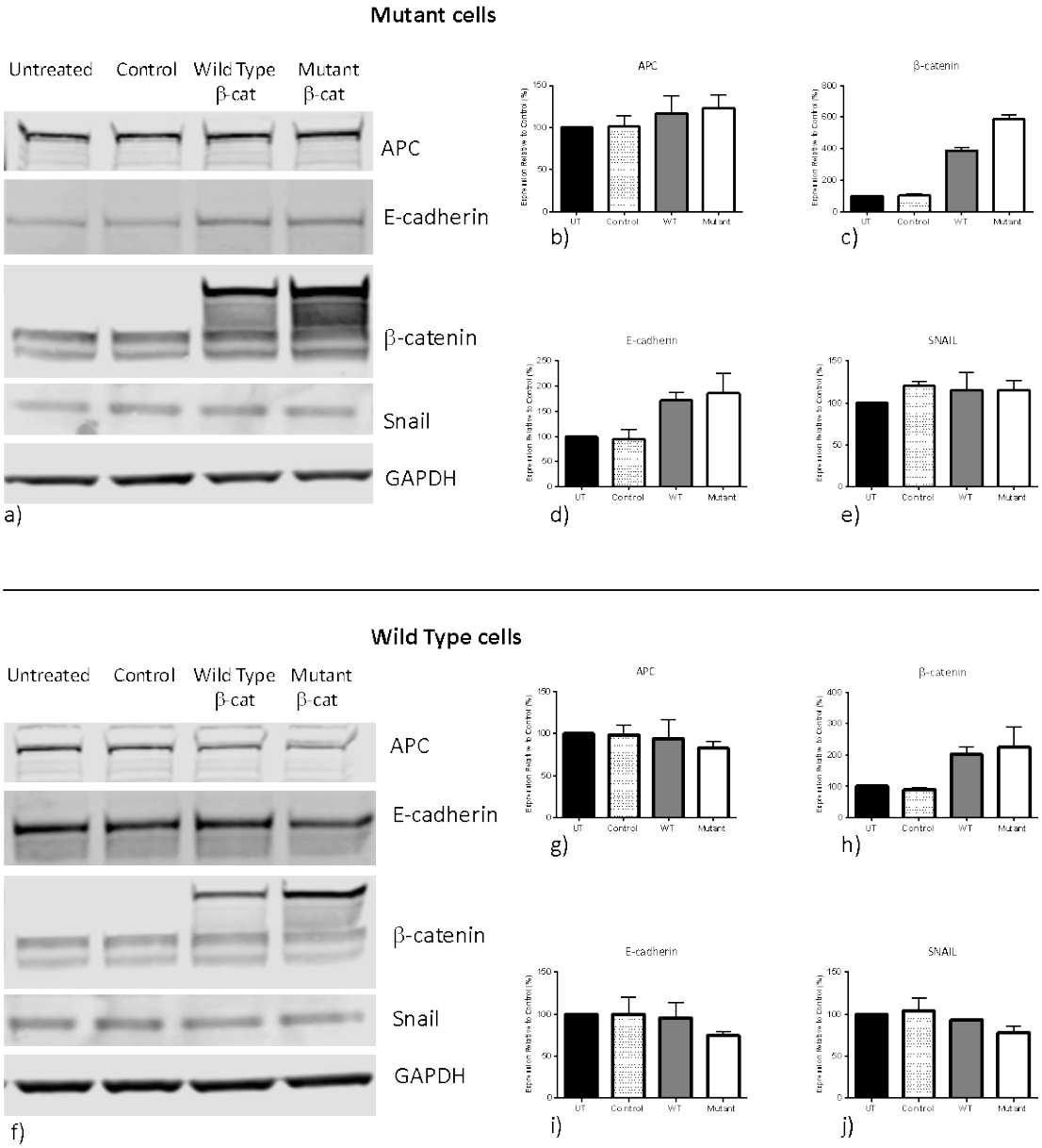



*Figure 3.14: Expression of exogenous mutant and wild-type β-catenin proteins causes a differential response in protein expression levels between wild-type and mutant HCT116 cells. Mutant (top panel) and wild-type (bottom panel) HCT116 cells were transfected with either mutant or wild-type β-catenin with a myc tag. Western blot of whole-cell lysate was probed with antibodies directed against APC, E-cadherin, β-catenin and GAPDH. Representative western blots of the mutant (a) and wild-type (b) cells are shown. Protein levels were quantified relative to GAPDH. Protein levels under the different treatment conditions are plotted in (b, c, d & e) for mutant cells and (g, h, I & j) for wild type. Data represent the mean and standard deviation of three independent experiments.*

An increase in the level of E-cadherin and β-catenin protein was measured following transfection of both the mutant and wild-type β-catenin in the mutant cell line. Only β-catenin levels rose in the wild-type cells.

In the mutant cell line, no significant differences in the levels of APC or Snail ($p>0.05$) were observed following transfection of either mutant or wild-type β-catenin, although a slight up-regulation of APC was noted. I measured the expression level of Snail because it has been implicated in the down-regulation of E-cadherin in the process of EMT. This transcriptional repression of E-cadherin by Snail can be caused by elevated β-catenin. A statistically significant rise in E-cadherin level was noted after transfection of both β-catenin proteins ($p<0.05$). This could potentially relate to stabilisation of the E-cadherin protein by the extra β-catenin or could represent transcription of new E-cadherin. The increase in E-cadherin protein may function to bind some of the exogenous β-catenin. This may be an attempt to control levels of free β-catenin as the APC in the mutant cells may be saturated with sequestered non-degradable β-catenin. Any rise in E-cadherin in the mutant cell line may be especially noticeable as baseline levels are very low under control conditions.

No significant differences in the expression levels of any protein in response to transfection of either β-catenin were measured in the wild-type cell line ($p>0.05$). The wild-type cells' capability to degrade or sequester β-catenin may be sufficient to regulate levels of free β-catenin without an increase in either APC or E-cadherin. This hypothesis is outlined schematically in Figure 3.15.

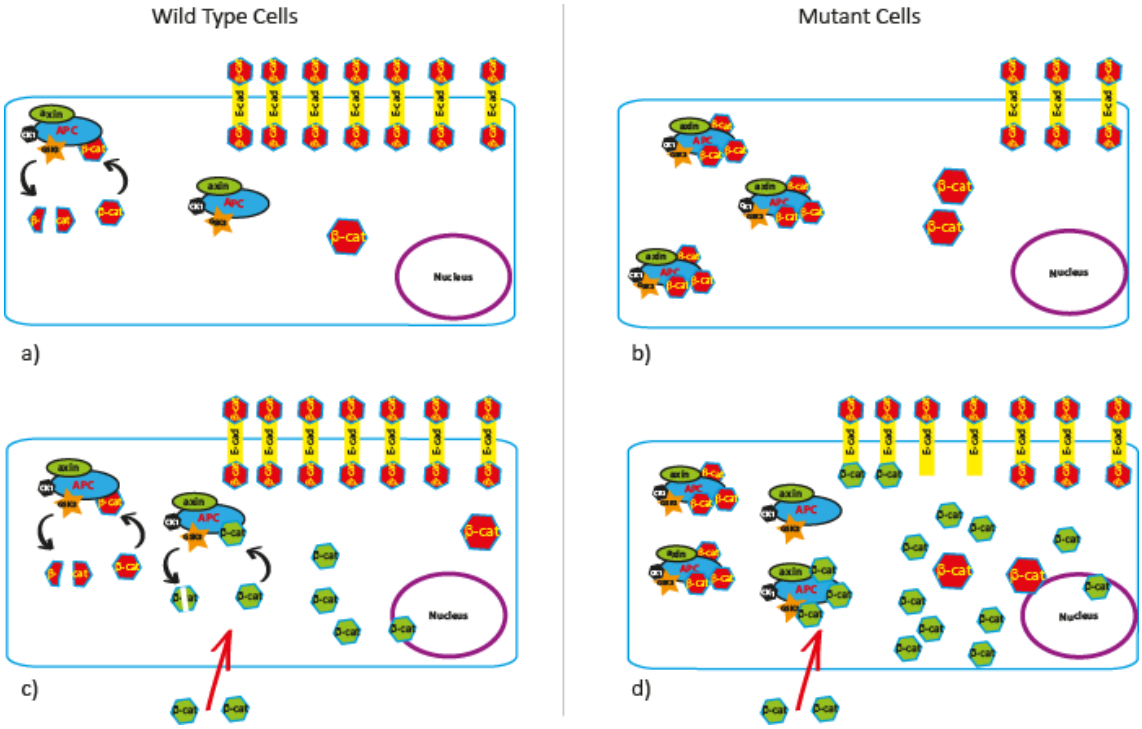



*Figure 3.15: Wild-type (a) and mutant cells (b) are shown under control conditions. Wild-type cells express more E-cadherin than the mutant cells and β-catenin can undergo degradation by the APC destruction complex. The mutant cells express little E-cadherin and the β-catenin is resistant to degradation, but can be sequestered by APC. Upon transfection of exogenous β-catenin (green), the wild-type cells (c) can respond by degrading both endogenous and exogenous wild-type β-catenin using available APC. This function is possible without additional APC or E-cadherin. Transfection of the mutant β-catenin causes a larger rise in β-catenin in the wild-type cells. This may be explained by APC binding sites becoming occupied in the sequestration of the mutant β-catenin. The mutant cell line destruction complex can sequester but not degrade endogenous β-catenin. This may reduce the number of available APC binding sites for exogenously expressed β-catenin. In addition, the mutant cells have low levels of E-cadherin. Upregulation of APC and E-cadherin (d) in response to exogenous β-catenin aims to limit the magnitude of the free unbound poll of β-catenin.*

I next determined where in the cell exogenously expressed β-catenin localised and whether this impacted on the localisation of endogenous β-catenin or E-cadherin (Figure 3.16). The relative proportional distribution between the Cytoplasm, Membrane, Nucleus and cytoskeleton was assessed. The exogenous β-catenin could be identified by its greater molecular weight in comparison to endogenous β-catenin due to the presence of a myc tag.

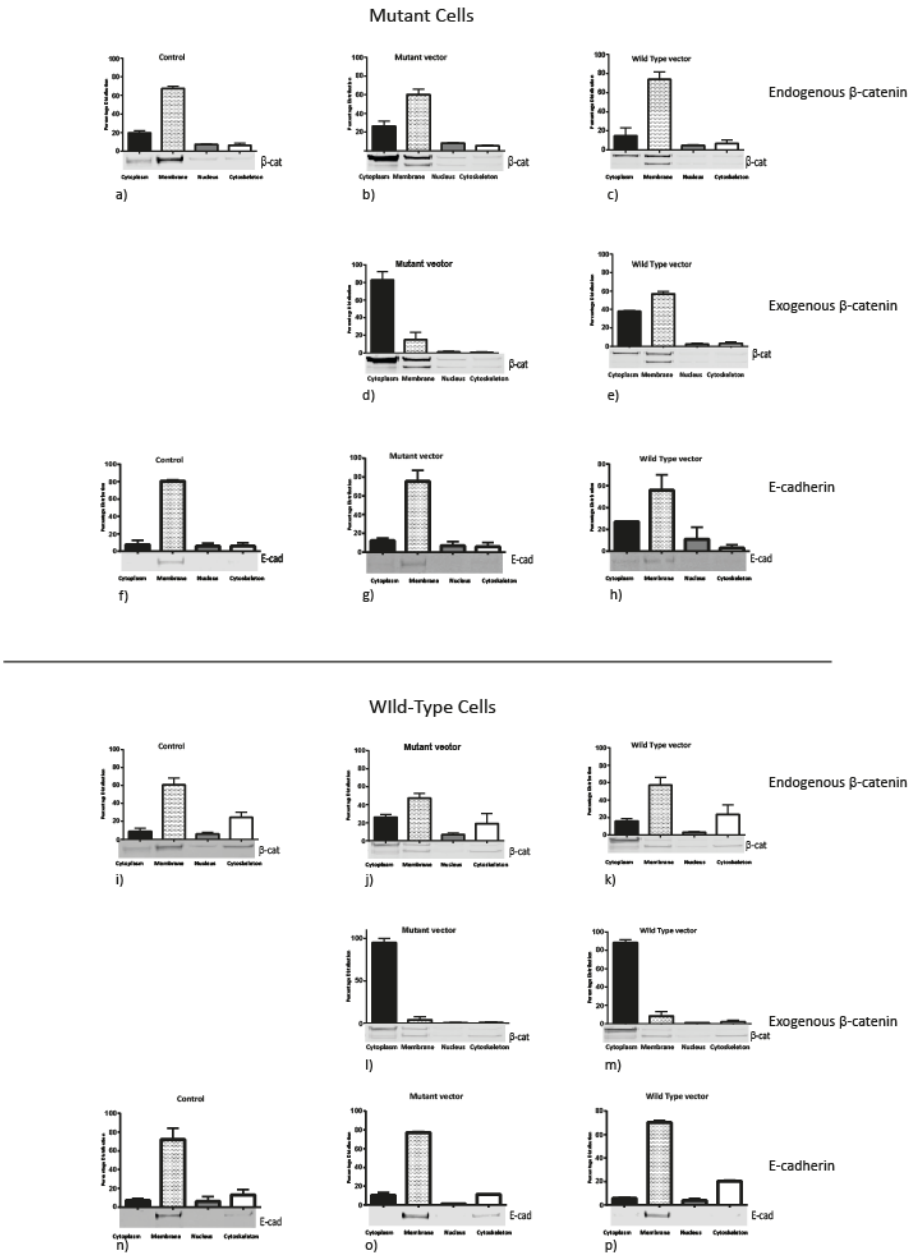



*Figure 3.16: Exogenously expressed mutant or wild-type β-catenin predominantly localises in the cytoplasm but does not change the localisation of endogenous β-catenin or E-cadherin. Mutant (top panel) and wild-type (bottom panel) HCT116 cells were transfected with either mutant or wild-type β-catenin and fractionated into cytoplasm, membrane, nuclear and cytoskeletal compartments. The proportional localisation of both endogenous and exogenous β-catenin was measured using western blot with antibodies against β-catenin and E-cadherin. The proportional distribution of endogenous β-catenin was measured by quantification of the lower β-catenin band and the mean and standard deviation were plotted in (b &c) for the mutant and (j & k) for the wild-type cells. The proportional localisation of exogenous β-catenin was measured by quantification of the upper β-catenin band and plotted in (d & e) for mutant and (l & m) for wild-type cells. E-cadherin distribution was quantified and plotted in (f, g, h, n, o & p). Blot of E-cadherin following transection was done only once.*

The distribution of β-catenin in both the mutant and wild-type cells does not change significantly following transfection of either mutant or wild-type β-catenin. Similarly, no change in the pattern of E-cadherin distribution was measured. Exogenous β-catenin predominantly localises in the cytoplasm of the wild-type cell lines following transfection of either mutant or wild-type β-catenin. The exogenous β-catenin also shows a shift to the cytoplasm in the mutant cells but some exogenous wild-type β-catenin localises to the membrane in these cells.

No dramatic change in the localisation of endogenous β-catenin is measured following transfection of exogenous β-catenin in either cell line. This suggests that the exogenous β-catenin does not competitively move endogenous β-catenin from its binding sites during the time course of this experiment.

In the wild-type cells the exogenous β-catenin localised largely in the cytoplasm, suggesting it was free and unbound. No increase in either APC or E-cadherin levels was noted in these cells following transfection that may have increased the number of binding partners for the exogenous β-catenin.

In the mutant cells, the mutant exogenous β-catenin localises predominantly in the cytoplasm. The wild-type exogenous β-catenin, however, is located at the membrane. The differences in localisation between the two exogenous β-catenin proteins in these cells may indicate that the mutant β-catenin cannot interact as well with E-cadherin as does the wild type.

Having determined the localisation of exogenous β-catenin, I next aimed to measure its impact on transcription of AXIN2 and E-cadherin (Figure 3.17).

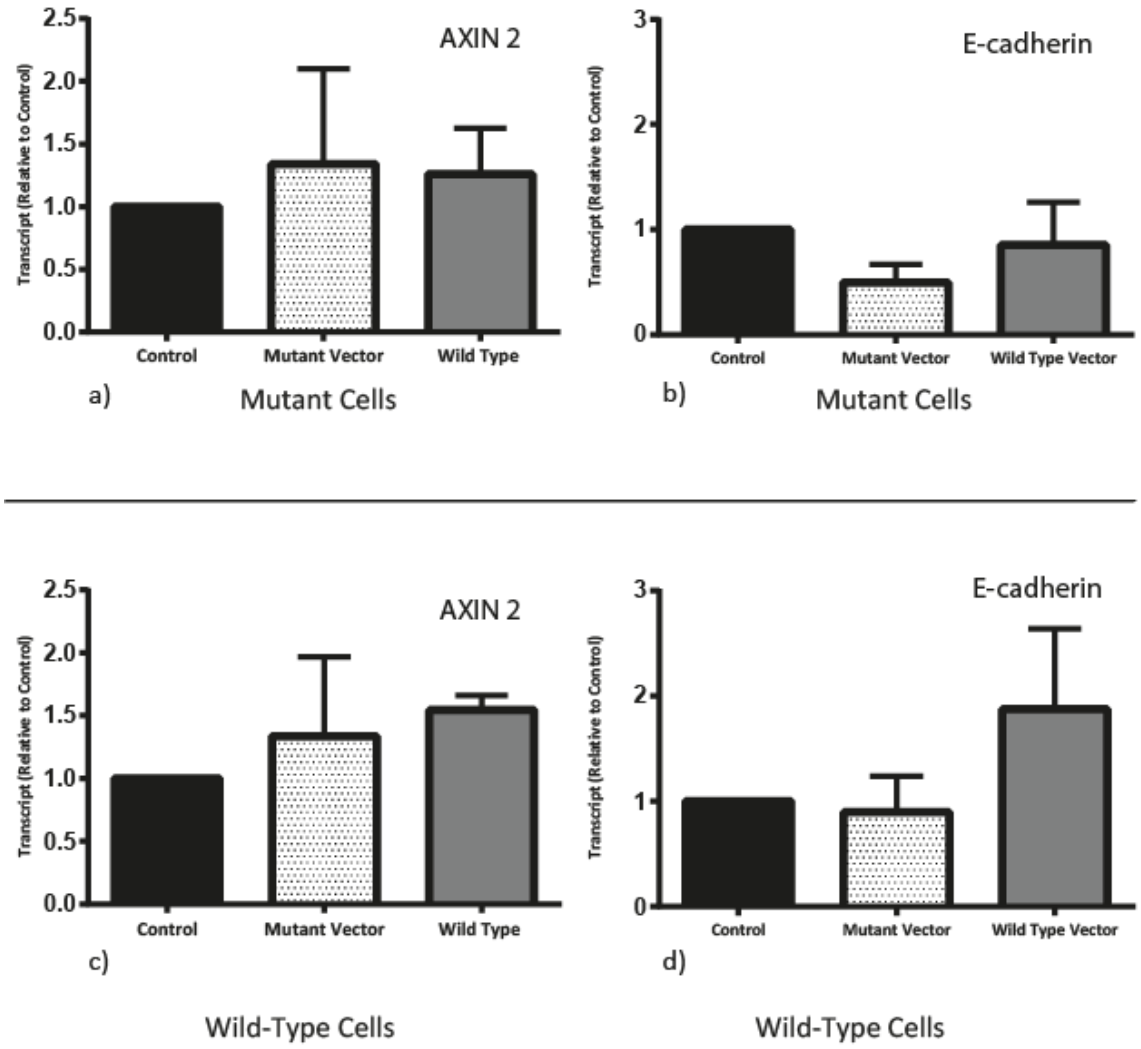



*Figure 3.17: Over-expression of β-catenin causes an increase in AXIN2 transcription in both mutant and wild-type HCT116 cells, while the effect on E-cadherin is variable. Wild-type and mutant HCT116 cells were transfected with either mutant or wild-type β-catenin. Cell lysates were processed and analysed for levels of AXIN2 and E-cadherin transcription using qPCR. Data represent the means and standard deviations of two independent experiments and transcript levels are plotted relative to the control (empty vector) for mutant (a & b) and wild-type cells (c & d).*

Over-expression of both mutant and wild-type β-catenin caused an increase in the transcription of AXIN2 in both cell lines. E-cadherin transcript reduced slightly following transfection of the mutant β-catenin in the mutant cells and increased in the wild-type cells following transfection of the wild-type β-catenin.

AXIN2 transcription increased in both cell lines in response to transfection of both mutant and wild-type exogenous β-catenin. This finding is consistent with the increased levels of β-catenin following transfection. It also supports previous findings that associated a cytoplasmic localisation of β-catenin with an increase in AXIN2 transcription.

E-cadherin protein levels increased in the mutant cell line following transfection of both mutant and wild-type β-catenin. Transcript levels did not reflect this and E-cadherin transcript was reduced following transfection of the mutant β-catenin. This indicates that the increase in protein level may have been due to stabilisation.

E-cadherin transcript level increased in the wild-type cells following transfection of wild-type β-catenin. This contradicts findings at the protein level, which did not change significantly. Reasons for this are unclear and further experiments are required for clarification.

### 3.2.5 Summary of Key Findings:

- Overexpression of both mutant and wild-type β-catenin protein causes a rise in E-cadherin protein in cells expressing stabilised endogenous β-catenin.
- Exogenous β-catenin localises to the cytoplasm in the wild-type cells but some wild-type exogenous β-catenin is retained at the membrane in the mutant cell.
- Expression of exogenous β-catenin stimulates an increase in AXIN2 transcription in both mutant and wild-type cells.

## 3.3 Discussion

In this chapter, I aimed to determine how a point mutation causing stabilisation of β-catenin affects its interaction with APC and E-cadherin. I have established that stabilised β-catenin has increased binding to APC and this serves to sequester stabilised β-catenin. This affects the

transcriptional output of β-catenin as measured by AXIN2 transcription. Despite the mutant cell line having higher levels of β-catenin and very low levels of E-cadherin compared with the wild-type cells, AXIN2 transcription does not differ significantly between the cell lines. This indicates that sequestration of β-catenin by APC can limit the transcriptional function of β-catenin.

Depletion of APC in all cell lines stimulated a rise in AXIN2 transcription. This was despite only modest increases in total β-catenin level in the parental and wild-type cells and no change in the mutant cells. A redistribution of β-catenin to the cytoplasm followed APC depletion in all cell lines. This signifies that loss of both degradative and sequestration functions can stimulate an increase in the transcriptional output of β-catenin by not only impacting on total levels, but also on the localisation of β-catenin within the cell. I believe this cytoplasmic pool of β-catenin to be "free", unbound, and able to activate transcription. These data highlight the importance of both the presence and functional integrity of APC and E-cadherin in the regulation of β-catenin to maintain its "free" levels and transcriptional output within acceptable limits. These results also raise some potential insights into how tumour behaviour may differ even if the molecular defects appear to be similar, at least on the surface. APC loss in tumours results in the lack of ability to degrade β-catenin, which becomes stabilised and can activate a transcriptional programme. A point mutation causing stabilisation of β-catenin also results in the loss of ability to degrade β-catenin, but the functional consequences of this are different. Point mutated β-catenin can be sequestered by APC, thus minimising transcriptional activation. As long as subsequent mutations are not acquired, this mutation may have reasonably benign consequences for the cell and may not be enough to initiate or progress neoplastic change in isolation. This may partly explain why tumours with stabilising β-catenin mutations are far less common than those with APC loss.

I also established that cells expressing stabilised β-catenin have very low levels of E-cadherin protein and transcript compared with wild-type cells. It is unclear if the high level of interaction

between stabilised β-catenin and APC results in a lack of β-catenin being available to bind to E-cadherin. This scenario could mean that there is no requirement for a high level of E-cadherin as there is a limited pool of β-catenin available to bind. Alternatively, the stabilised β-catenin may not be able to bind to E-cadherin as efficiently as the wild-type protein. I attempted to clarify this by over-expressing mutant and wild-type β-catenin in both cell lines. I had expected responsive increases in E-cadherin to accommodate the rise in β-catenin. Expression of the wild-type β-catenin in the mutant cell line caused it to localise mainly at the membrane with some in the cytoplasm. This could signify that the wild-type protein is able to interact with E-cadherin more efficiently than the mutant protein, which favoured a cytoplasmic localisation. Exogenous mutant and wild-type β-catenin both localised to the cytoplasm in the wild-type cells. Co-IP studies identified a high level of interaction between β-catenin and E-cadherin. This may indicate that E-cadherin is saturated and given that no increase in E-cadherin was observed following over-expression, the β-catenin may have localised in the cytoplasm due to the lack of available E-cadherin binding sites. Further co-IP studies of β-catenin and E-cadherin following transfection of exogenous β-catenin may help to clarify these relationships.

I included analysis of the effects of depleting PTEN on the levels of APC, β-catenin and E-cadherin. In all cell lines, PTEN knockdown caused significant increases in levels of E-cadherin protein and transcript. Modest increases in β-catenin were measured under these conditions that may be due to activation of the Akt pathway, which can inhibit GSK3β. This would negatively impact on the function of the APC destruction complex. The rise in β-catenin may cause some stabilisation of E-cadherin, contributing to the observed rise, but the increase in E-cadherin transcript signifies that new E-cadherin is likely to be the main contributor to the increase. The reasons for increased E-cadherin transcription following PTEN depletion are unclear and require further experiments for clarification. The findings are consistent between all cell lines studied, signifying that the mechanism is independent of whether stabilised or wild-type β-catenin is expressed.

# 4 The influence of PTEN on the interactions of β-catenin, APC and E-cadherin

## 4.1 Introduction

PTEN is a multifunctional protein with an important role as a tumour suppressor. It plays a key role in the negative regulation of the Akt signalling pathway. Activation of the Akt pathway follows PTEN loss, stimulating cell proliferation, progression of the cell cycle, growth and survival. Homeostasis of these functions are critical for normal gut function and defects in these are linked to cancer.(Song, 2012, Koul, 2002, Fata, 2012) (Figure 1.9). PTEN is also implicated in crohns disease. Reactive oxygen species are thought to be able to activate nuclear NF-kB via the PTEN/Akt pathway leading to inflammation linked to crohns (Tokuhira *et al.* 2015)

Potential crosstalk between PTEN and the adhesion junctional complex of β-catenin and E-cadherin also exists. PTEN and PI3-kinase have been identified in association with E-cadherin and β-catenin at the adherens junctions (Vogelmann, 2005) and PTEN has been detected in co-immunoprecipitates of both E-cadherin and β-catenin in MDCK cells (Kotelevets, 2005)

In the previous chapter, Ilammatio found that a stabilising mutation in β-catenin can affect its interaction with APC and E-cadherin. In addition, I measured the impact of PTEN depletion on the levels of these three proteins. I found that PTEN knockdown caused significant increases in the level of E-cadherin transcript and protein irrespective of whether the cell expressed wild-type or stabilised β-catenin.

Analysis of the effects of PTEN were included because PTEN loss is commonly implicated in colorectal cancer and has been associated with tumours developing on the serrated pathway of colorectal carcinogenesis (Day *et al.* 2013, Davies *et al.* 2014). In addition, PTEN defects are also found in tumours developing on the conventional pathway of colorectal cancer, which are

characterised by APC mutations. I aimed to determine if there was potential crosstalk between these two pathways. Experiments in Chapter 1 have shown a rise in E-cadherin at both the transcriptional and protein level following PTEN loss in HCT116 colorectal cancer cells. In conjunction with APC, E-cadherin plays a key role in the regulation of β-catenin. Disturbances in the balance of how β-catenin interacts with APC and E-cadherin are central to the development of colorectal cancer and usually occur following loss of APC. I aimed to explore further how loss of one gene commonly implicated in cancer, PTEN, could impact on E-cadherin, a protein tightly linked to the important tumour suppressor gene, APC.

In this chapter I aim to:

1) Compare total protein levels of APC, β-catenin and E-cadherin in PTEN (+/+) and PTEN (-/-) cells.
2) Measure transcript levels of β-catenin, E-cadherin and AXIN2 in these two cell lines.
3) Establish if PTEN status affects the localisation of β-catenin and if this changes in response to depletion of APC, β-catenin, E-cadherin or PTEN.
4) Determine how PTEN affects the relative level of interaction between β-catenin in complexes containing APC and those containing E-cadherin.
5) Establish if expression of PTEN proteins in PTEN (-/-) cells can cause changes in protein level of APC, β-catenin and E-cadherin or affect transcription of AXIN2 and E-cadherin.

## 4.2 Results

### 4.2.1 PTEN loss affects levels of APC, β-catenin and E-cadherin in HCT116 cells

I first measured if PTEN null cells displayed a different expression profile of APC, β-catenin and E-cadherin proteins using HCT116 human colorectal cancer cells. I compared two cell lines. First, a PTEN wild-type cell line PTEN (+/+) that expresses one wild-type β-catenin allele (+) and one mutant (Δ45) allele (+/Δ45). The mutant allele renders the β-catenin protein non-degradable by the APC destruction complex due to deletion of reside serine 45 (Δ serine 45). The second cell line is a PTEN null cell PTEN (-/-) with the same complement of β-catenin as the PTEN (+/+) cell line. The cells are genetically identical other than the differences in PTEN. Cell lysis and western blot analyses (Figure 4.1) showed differential expression of APC, β-catenin and E-cadherin between the cell lines studied.

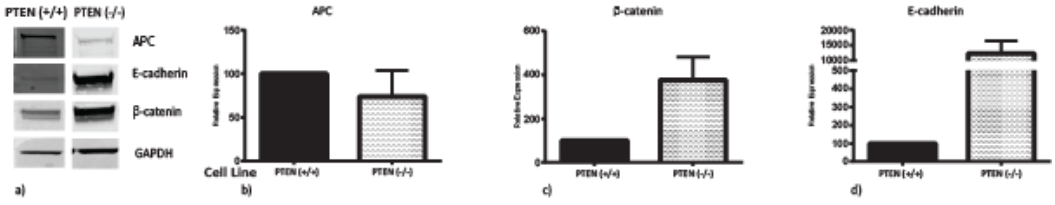



*Figure 4.1: PTEN status influences the levels of APC, β-catenin and E-cadherin. Whole-cell lysate of PTEN (+/+) and PTEN (-/-) cells were subjected to western blot with antibodies against APC, β-catenin and E-cadherin. Protein levels were quantified relative to GAPDH. Plots show protein levels APC in (a), β-catenin in (b) and E-cadherin in (c) plotted relative to the level in the PTEN (+/+) cells. Data represent the mean and standard deviation of three independent experiments.*

PTEN (-/-) cells express more β-catenin and E-cadherin than PTEN (+/+) cells. No difference exists in the expression levels of APC.

PTEN (-/-) cells have higher levels of β-catenin. This could potentially be due to activation of the Akt pathway, which can inhibit GSK3β and negatively influence the function of the β-catenin destruction complex (Song, 2012, Koul, 2002, Fata, 2012) (Figure 4.2b.)

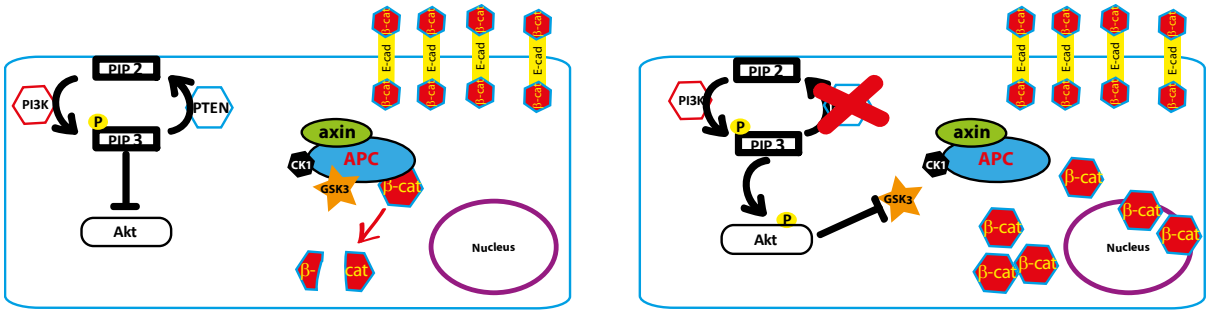



*Figure 4.2: A simplified representation of the PTEN Akt pathway and its potential mechanism of influence on β-catenin level. Cell a) represents the phosphorylation of PIP2 to PIP3 by PIP3 kinase and its subsequent dephosphorylation by PTEN. This results in unphosphorylated Akt, uninhibited GSK3β and an APC destruction complex with normal β-catenin degradative function. Cell b) signifies PTEN loss, phosphorylation of Akt with resultant inhibition of GSK3β. This negatively impacts on the function of the APC destruction complex and can allow accumulation of β-catenin.*

The higher levels of E-cadherin in PTEN (-/-) cells support findings in Chapter 1 that showed PTEN depletion resulted in an increase in both E-cadherin transcript and protein level.

The PTEN (+/+) and PTEN (-/-) cells express the same forms of β-catenin. Some can be degraded by APC and some will be sequestered by APC in both cell lines. However, the PTEN (-/-) cells contain much more total β-catenin. Perhaps the additional β-catenin saturates the ability of APC to regulate it (for instance because too much mutant β-catenin is stably associated with APC). The resulting excess may be too much to be sequestered or controlled so that once a threshold is exceeded, more E-cadherin is expressed that can sequester this β-catenin to regulate the amount of free β-catenin in the cell (Figure 4.3).

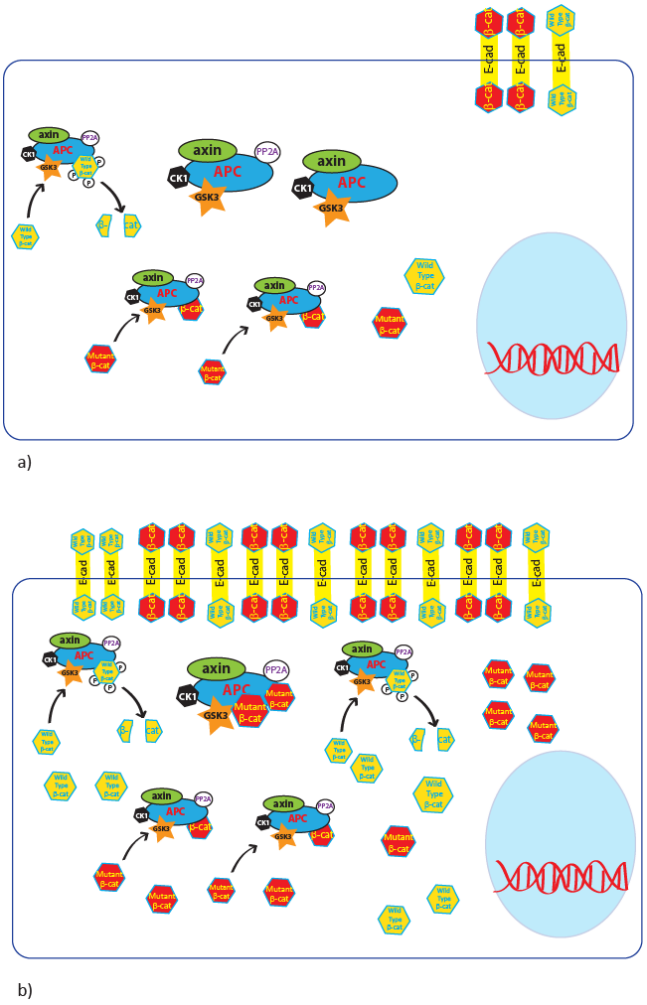



*Figure 4.3: In PTEN (+/+) cells (a) β-catenin levels are lower and can therefore be either degraded (Wild Type (yellow)) or sequestered (Mutant (red)) by APC to maintain free cytoplasmic levels within reasonable limits. Only a small amount of E-cadherin is required to sequester some of this β-catenin at adherens junctions. PTEN (-/-) cells (b) have far more β-catenin. This may saturate the capabilities for APC to either sequester or degrade, resulting in increased free β-catenin in the cytoplasm. An unknown mechanism may upregulate E-cadherin to bind this free β-catenin at the plasma membrane.*

To test whether the effects of PTEN loss on β-catenin and E-cadherin related to protein stability or increased transcription, I measured transcript levels of β-catenin and E-cadherin. In addition, I measured transcription of AXIN2, a β-catenin target gene (Figure 4.4).

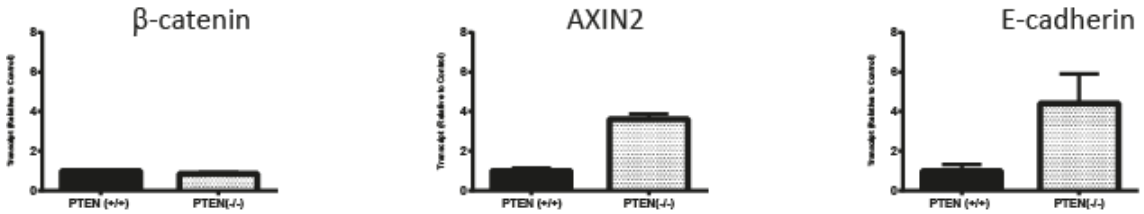



*Figure 4.4: Differences exist in the transcription of AXIN2 and E-cadherin between PTEN (+/+) and PTEN (-/-) cells while β-catenin transcription is similar. Transcript levels of β-catenin (a), AXIN2 (b) and E-cadherin (c) were assessed on whole-cell lysate by qPCR. Plotted data show the transcript level relative to the PTEN (+/+) cells and represent the mean and standard deviation of three independent experiments. Plotted values represent the mean and standard deviation of three independent experiments for each condition.*

No significant difference was measured between transcript levels of β-catenin in PTEN (+/+) and PTEN (-/-) cells. Transcription of AXIN2 and E-cadherin was significantly higher in PTEN (-/-) cells compared with PTEN (+/+) cells.

This indicates that the excess β-catenin protein in the PTEN (-/-) cells is not the result of increased transcription but likely due to increased protein stability caused by decreased degradation.

E-cadherin transcription is much higher in the PTEN (-/-) than in the PTEN (+/+) cells. This indicates that the higher E-cadherin protein level in PTEN (-/-) cells is at least in part due to increased transcription rather than stabilisation of the E-cadherin protein. Whether higher E-cadherin transcription is a direct response to higher β-catenin levels is unclear.

AXIN2 transcript level is approximately four times higher in the PTEN (-/-) cells than in the PTEN (+/+) cell line. The β-catenin protein level is also in the region of four times higher in the PTEN (-/-) cells than in the PTEN (+/+) cells. This finding could simply reflect that the higher level of β-catenin present in the PTEN (-/-) cells stimulates AXIN2 transcription.

**4.2.1 Summary of Key Findings:**

- PTEN (-/-) cells have more β-catenin and E-cadherin protein than PTEN (+/+) cells.
- PTEN (+/+) and PTEN (-/-) cells have a similar transcript level of β-catenin but PTEN (-/-) cells have a higher transcript level of both E-cadherin and AXIN2.

**4.2.2.1 β-catenin, APC and E-cadherin are responsive to depletion of each other in PTEN (-/-) cells.**

Figure 4.1 revealed that PTEN can affect the total level of APC and E-cadherin in HCT116 cells. I next wanted to provide further insight into the relationships between APC, β-catenin and E-cadherin, and determine how they are influenced by PTEN. I compared levels of β-catenin, APC and E-cadherin after depletion of each of these proteins in PTEN (+/+) and PTEN (-/-) HCT116 cells (Figure 4.5).

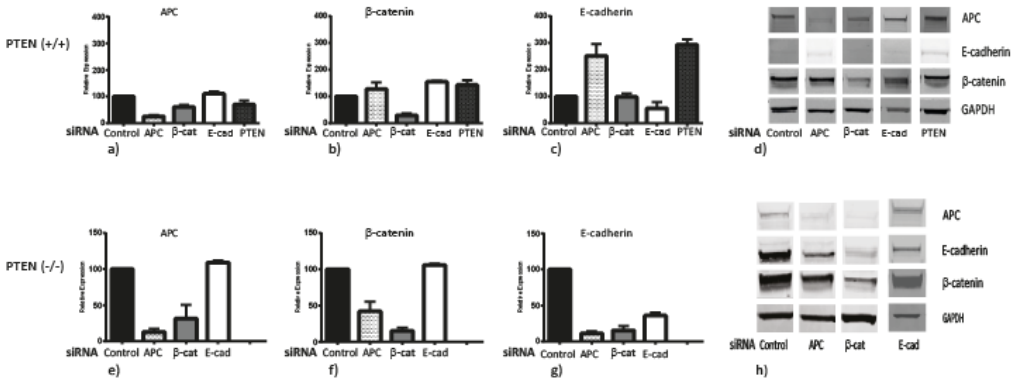



*Figure 4.5: Depletion of APC, β-catenin, E-cadherin or PTEN can influence expression levels of APC, β-catenin and E-cadherin in PTEN (+/+) and PTEN (-/-) HCT116 cells. Cells were treated with the appropriate siRNA then whole-cell lysate was subjected to western blot with antibodies directed against APC, β-catenin or E-cadherin. Protein levels were quantified relative to GAPDH. Protein levels of APC (a & e), β-catenin (b & f) and E-cadherin (c & g) are plotted relative to the control levels in PTEN (+/+) cells (top panel) and PTEN (-/-) cells (bottom panel). Representative western blots are shown in (d & h). Measurements represent the mean and standard deviation of three separate experiments and representative western blots are displayed for each condition.*

E-cadherin levels increase following PTEN depletion in PTEN (+/+) cells and after APC depletion. Levels of APC and β-catenin decrease following depletion of APC and β-catenin while E-cadherin levels are lower following all treatments in PTEN (-/-) cells.

The increase in E-cadherin following PTEN depletion is in keeping with the higher level of E-cadherin expression in PTEN (-/-) cells. The causative mechanism for this is unclear and requires further experiments. These data should be interpreted with caution as very low levels of E-cadherin are expressed in these cells. This may have the effect of amplifying even small changes due to measurement artefacts.

The APC level decreased in both cell lines following β-catenin depletion. This supports findings in Chapter 3 and appears to be independent of PTEN status. This may represent part of a feedback loop between APC and β-catenin.

There was a reduction in E-cadherin level following all treatments in the PTEN (-/-) cells, which contrasts with findings in the PTEN (+/+) cells. The reasons for this are not apparent from these experiments.

#### 4.2.2.2 Depletion of APC, β-catenin and E-cadherin causes differential effects on transcription of β-catenin, E-cadherin and AXIN2 in PTEN (+/+) and PTEN (-/-) cells

Next, I wanted to assess if transcript levels of β-catenin and E-cadherin reflected the changes in protein levels following depletion of APC, β-catenin, E-cadherin and PTEN in PTEN (+/+) and PTEN (-/-) HCT116 cells. In addition, I aimed to assess the impact of these treatments on the transcription of β-catenin target gene AXIN2 (Figure 4.6).

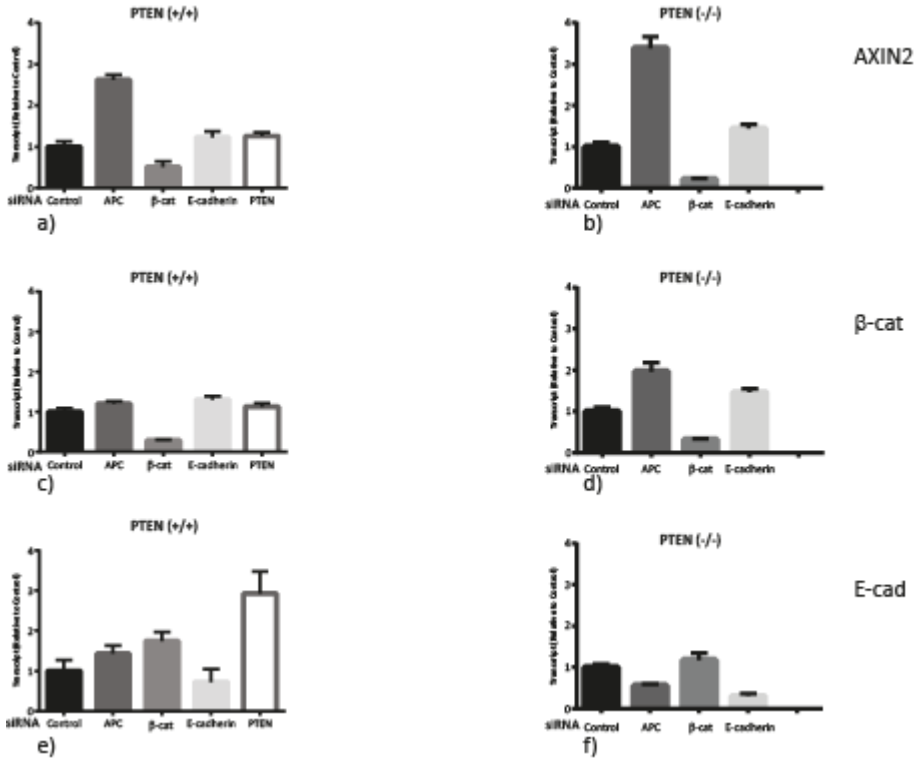



*Figure 4.6: Transcript levels of AXIN2, β-catenin and E-cadherin are differentially responsive to depletion of APC and PTEN in PTEN (+/+) and PTEN (-/-) cells. Cells were treated with siRNA against APC, β-catenin, E-cadherin or PTEN, then the transcript level of AXIN2 (a & b), β-catenin (c & d) and E-cadherin (e & f) was measured using qPCR. Transcript levels are plotted relative to a control. Data represent the mean and standard deviation of three independent experiments.*

AXIN2 transcription increases significantly following APC depletion in both cell lines. β-Catenin transcript increases following APC depletion in PTEN (-/-) cells and to a lesser extent in PTEN (+/+) cells under the same conditions. E-cadherin transcript level increases in PTEN (+/+) cells following PTEN depletion.

AXIN2 transcription increases in both cell lines in response to APC depletion. This supports the findings in Chapter 3. This is most likely due to loss of degradation of the wild-type β-catenin in these cells and release of the sequestered mutant β-catenin in both cell lines.

This may be supported by some new transcription of β-catenin, which is shown following APC depletion, especially in the PTEN (-/-) cells. The increased β-catenin transcript does not reflect the decrease in β-catenin protein in the PTEN (-/-) cells following APC depletion. The modest

increase in transcript in the PTEN (+/+) cells supports a similar modest increase in β-catenin protein in PTEN (+/+) cells after APC knockdown.

The increased in E-cadherin protein in PTEN (+/+) cells after PTEN knockdown is supported by an increase in E-cadherin transcript level. This indicates that new transcription is responsible for the increased expression levels as opposed to stabilisation.

**4.2 Summary of Key Findings:**

- Both E-cadherin protein and transcript increase in PTEN (+/+) cells following PTEN knockdown.
- AXIN2 transcription increases in both PTEN (+/+) and PTEN (-/-) cells following APC depletion.

#### 4.2.2.3 Depletion of APC and E-cadherin causes a redistribution of β-catenin from the plasma membrane to the cytoplasm

I next wanted to determine how depletion of APC, β-catenin, E-cadherin or PTEN affects the distribution of β-catenin in both PTEN (+/+) and PTEN (-/-) cells.

I fractionated cells into pools representing different cellular components: cytoplasm, membrane, nucleus and cytoskeleton, and measured the presence of β-catenin in each fraction. Graphically displayed data and representative blots are in Figure 4.7.

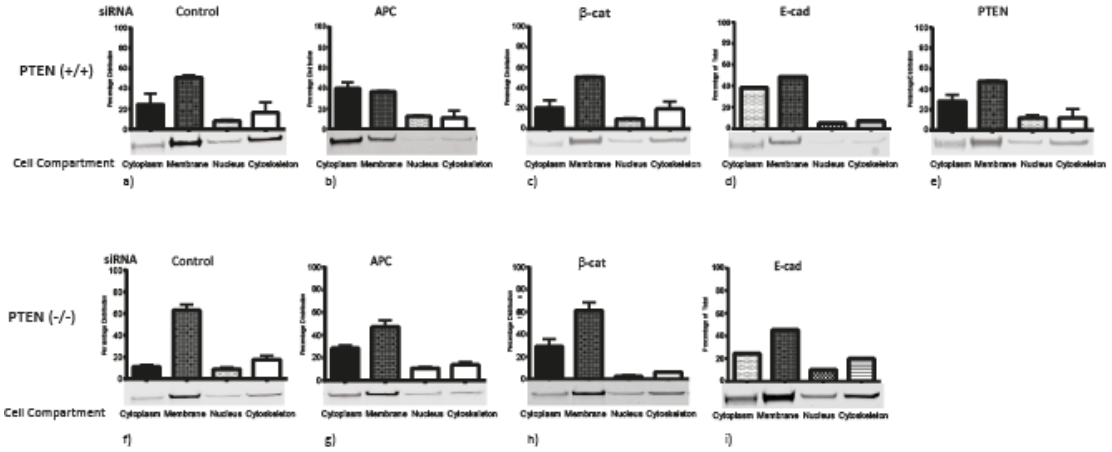



*Figure 4.7: Cellular location of β-catenin is altered by depletion of APC and E-cadherin in both cell lines studied. Cells were fractionated into cytoplasmic, membrane, nuclear and cytoskeletal compartments and localisation of β-catenin was assessed by western blot using antibody directed against β-catenin following depletion of APC (b & g), β-catenin (c & h), E-cadherin (d & i), PTEN (e) or under control conditions (a & f). Data represent the mean and standard deviation of three independent experiments, except E-cadherin knockdown, where n = 1.*

PTEN is predominantly located at the plasma membrane in both PTEN (+/+) and PTEN (-/-) cells. Depletion of APC or E-cadherin causes a redistribution of β-catenin to the cytoplasm in both cell lines.

The distribution of β-catenin under control conditions shows a similar trend in both cell lines. In the PTEN (+/+) cell line, approximately 50–60% of total β-catenin resides in the membrane

compartment of the cells with 20–30% in the cytoplasm. The cytoskeletal compartment contains approximately 20% of β-catenin, while nuclear β-catenin accounts for 10%. A similar distribution is found in the PTEN (-/-) cells. The main difference from the PTEN (+/+) cells is that around 65% of β-catenin is membrane associated at the expense of the cytoplasmic compartment, which has 15%. The difference between the membrane proportion in PTEN (+/+) compared with PTEN (-/-) cells was statistically significant *(*$p<0.05$*).*

Based on these figures, the greatest proportion of β-catenin appears to be associated with E-cadherin at the cell membrane under control conditions. Perhaps the much higher level of E-cadherin in the PTEN (-/-) cells can explain the higher proportion of β-catenin in the membrane region.

Depletion of APC causes a redistribution of β-catenin to the cytoplasm from the plasma membrane in both cell lines. This supports findings from Chapter 3. This is likely due to release of sequestered mutant β-catenin from APC and loss of degradation of wild-type β-catenin. The cytoplasmic β-catenin represents a free unbound pool.

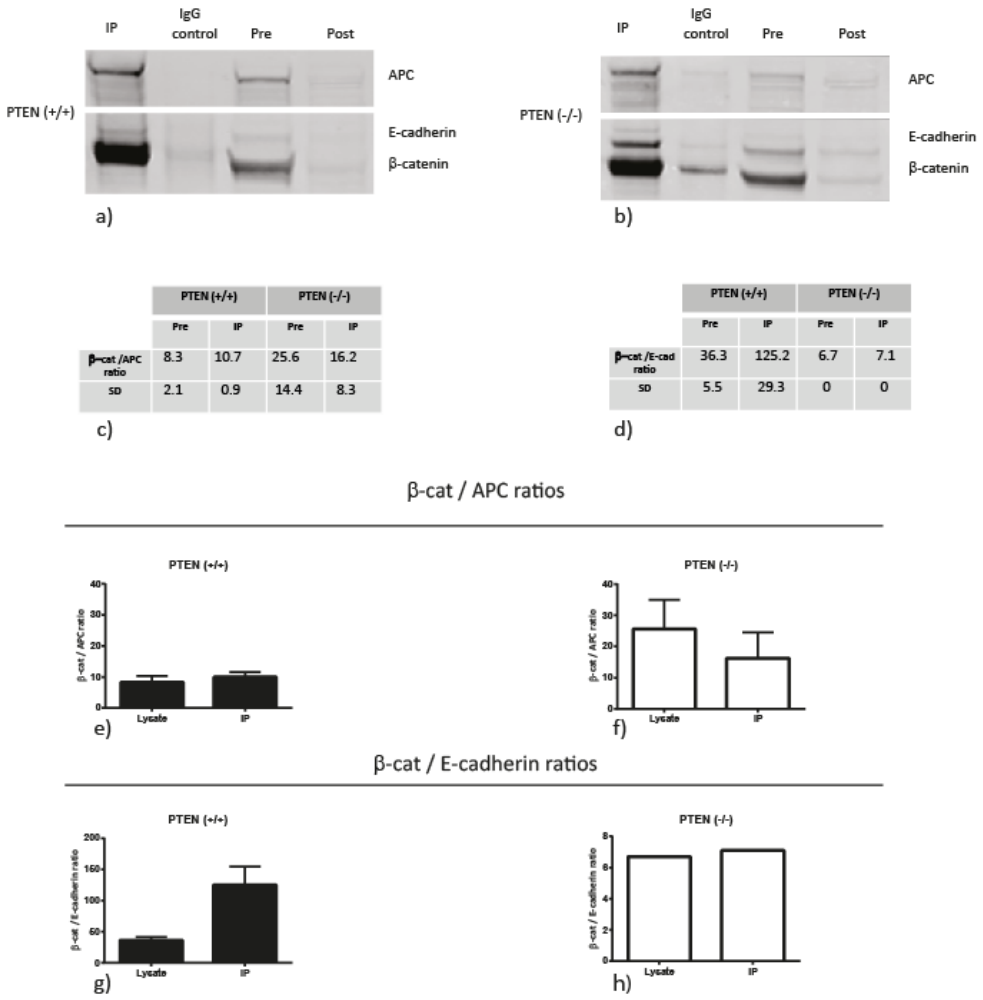
E-cadherin depletion causes a similar redistribution of β-catenin to the cytoplasm from the plasma membrane. This would be expected following dissociation of adherens junctions and occurred in both cell lines.

**4.3 Summary of Key Findings:**

- Significantly more β-catenin is located at the plasma membrane in PTEN (-/-) cells compared with PTEN (+/+) cells.
- Depletion of APC or E-cadherin results in a redistribution of β-catenin from the plasma membrane to the cytoplasm irrespective of PTEN status.

**4.4 The Association of β-Catenin with APC is Unaffected by PTEN Status While More β-Catenin is Bound to E-cadherin in PTEN Cells Compared with PTEN (+/+) Cells**

In Chapter 3, I showed that stabilising mutations of β-catenin can influence its relative interaction with APC or E-cadherin. Based on the effects of PTEN on E-cadherin in particular, I aim to assess if PTEN impacts on these relationships using β-catenin Co-IP (Figure 4.8).




| | PTEN (+/+) | | PTEN (-/-) | |
|---|---|---|---|---|
| | Pre | IP | Pre | IP |
| β-cat /APC ratio | 8.3 | 10.7 | 25.6 | 16.2 |
| SD | 2.1 | 0.9 | 14.4 | 8.3 |

| | PTEN (+/+) | | PTEN (-/-) | |
|---|---|---|---|---|
| | Pre | IP | Pre | IP |
| β-cat /E-cad ratio | 36.3 | 125.2 | 6.7 | 7.1 |
| SD | 5.5 | 29.3 | 0 | 0 |

*Figure 4.8: The distribution of β-catenin between complexes containing APC or E-cadherin is different in PTEN (+/+) cells compared with PTEN (-/-) cells. β-Catenin was co-immunoprecipitated from PTEN (+/+) and PTEN (-/-) cells. Western blot of the IP and lysate was probed with antibodies against APC, β-catenin and E-cadherin. Representative western blots of IP and lysate are shown for PTEN (+/+) (a) and PTEN (-/-) cells (b). Values of β-catenin/APC are tabulated (c) and plotted (e) for PTEN (+/+) and (f) for PTEN (-/-) cells. β-catenin/E-cadherin ratios are tabulated in (d) and plotted in (g) for PTEN (+/+) and (h) for PTEN (-/-) cells. Data represent the mean and standard deviation of three independent experiments.*

The association of β-catenin with APC is not significantly different between the PTEN (+/+) and PTEN (-/-) cells ($p = 0.324$). More β-catenin is found associated with E-cadherin in the PTEN (-/-) cells compared with PTEN (+/+) cells.

A similar level of β-catenin binds to APC in the PTEN (+/+) compared with the PTEN (-/-) cells reflected by β-catenin/APC ratios of 10.7 and 16.2. Both these figures are similar to the β-catenin/APC ratios in the lysate of these cells (8.3 in PTEN (+/+) and 25 in PTEN (-/-)). A similar degree of APB/β-catenin interaction may be expected as both cell lines express one mutant and one wild-type β-catenin protein.

Significantly more β-catenin is found in association with E-cadherin in the PTEN (-/-) compared with the PTEN (+/+) cells reflected by β-catenin/E-cadherin ratios of 7.1 and 125.2 in the IP respectively ($p < 0.01$). The higher level of interaction may simply reflect the fact that the PTEN (-/-) cells have more β-catenin and E-cadherin, so there is more opportunity for binding.

To investigate these relationships further, I also immunoprecipitated APC and measured the amount of β-catenin that was recovered in the different cell types (Figure 4.9).

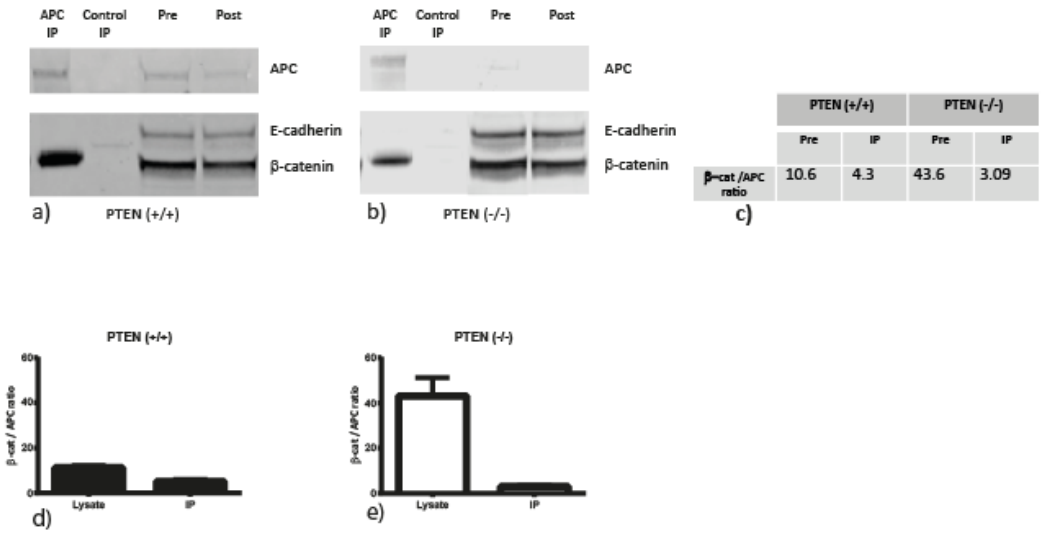
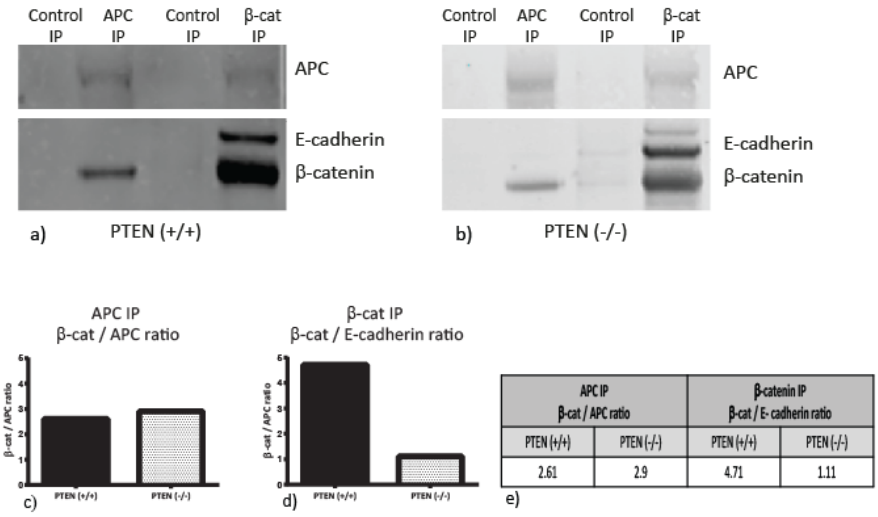



*Figure 4.9: The level of β-catenin in complexes containing APC or E-cadherin differs between PTEN (+/+) cells and PTEN (-/-) cells with more β-catenin associated with E-cadherin in PTEN (-/-) cells. APC Co-IP using APC-ALI antibody was carried out in PTEN (+/+) and PTEN (-/-) cells. Western blot on the IP and lysate was performed with antibodies directed at APC and β-catenin. Representative blots of the PTEN (+/+) (a) and PTEN (-/-) (b) cells are shown. The β-catenin/APC ratios for the IP and lysate are tabulated in (c) and plotted for the PTEN (+/+) cells in (d) and PTEN (-/-) cells in (e). Data represent the mean and standard deviation of three independent experiments.*

A similar degree of interaction between β-catenin and APC is evident in PTEN (+/+) versus PTEN (-/-) cells reflected by similar β-catenin/APC ratios in the IP of 4.3 and 3.09, respectively. A greater difference in ratios determined from the lysate reflects the fact that the PTEN (-/-) cells have relatively more β-catenin and less APC than the PTEN (+/+) cells.

These findings support those of the β-catenin Co-IP and are in line with expectations of two cell lines expressing the same complement of β-catenin.

I next aimed to see if I could distinguish different pools of β-catenin bound to either APC or E-cadherin in the different cell lines. I first precipitated APC, and then precipitated β-catenin from the remaining, post-IP, lysates. This allowed me to determine if any or all of the β-catenin that remained after removing APC was bound to E-cadherin (Figure 4.10).




| APC IP β-cat / APC ratio | | β-catenin IP β-cat / E- cadherin ratio | |
|---|---|---|---|
| PTEN (+/+) | PTEN (-/-) | PTEN (+/+) | PTEN (-/-) |
| 2.61 | 2.9 | 4.71 | 1.11 |

*Figure 4.10: Removing APC-associated β-catenin reveals differential binding of β-catenin to E-cadherin in PTEN (+/+) and PTEN (-/-) cells. APC Co-IP was performed, and then the post-IP lysate was used for a β-catenin Co-IP. Western blot was carried out in PTEN (+/+) cells (a) and PTEN (-/-) cells (b) with antibody against APC, β-catenin and E-cadherin. Ratios of β-catenin/APC in the APC-Co-IP were calculated and plotted in (c). β-Catenin/E-cadherin ratios were calculated from the β-catenin Co-IP and plotted in (d). Tabulated data are in (e). One replicate of this experiment was performed.*

Similar levels of β-catenin are bound to APC in both cell lines. Significantly more β-catenin is associated with E-cadherin in the PTEN (-/-) compared with PTEN (+/+) cells.

These results give support to findings from the single APC immunoprecipitations described above. Similar ratios (2.61 in PTEN (+/+) and 2.9 in PTEN (-/-)) of β-catenin/APC were obtained in the APC IP. Although total levels of β-catenin and APC differ between the two cell lines, the proportion of interaction between APC and β-catenin is similar between the two cell lines. This interaction appears to function independently of PTEN status.

The ratios of β-catenin/E-cadherin in the subsequent β-catenin IP (4.71 in PTEN (+/+) and 1.1 in PTEN (-/-)) indicate that there is more β-catenin bound to E-cadherin in the PTEN (-/-) cells compared with PTEN (+/+) cells. This may relate to the fact that there are higher levels of both E-cadherin and β-catenin in the PTEN (-/-) cells (Figure 4.1).

**4.4 Summary of Key Findings:**

- Proportionately similar amounts of β-catenin bind to APC in PTEN (+/+) cells as in PTEN (-/-) cells.
- More β-catenin is found in association with E-cadherin in PTEN (-/-) cells compared with PTEN (+/+) cells. This may reflect the higher expression levels of these two proteins in PTEN (-/-) cells.

**4.5 Assessment of the Effects of Expression of PTEN Constructs in PTEN (-/-) Cells**

I determined that lack of PTEN in HCT116 cells leads to higher expression of both β-catenin and E-cadherin with an associated increased interaction between them. I next aimed to measure if reintroducing PTEN into PTEN (-/-) can cause changes in the levels of β-catenin or E-cadherin.

I transfected PTEN (-/-) cells with a panel of PTEN proteins that vary in the specific phosphatase functions of the expressed protein. This allows distinction of whether a specific catalytic function of the PTEN protein or its simple physical presence is involved in the effects observed.

I compared a wild-type PTEN vector, which encodes the full length PTEN protein with normal lipid and protein phosphatase function, with protein C124S, which encodes full length PTEN lacking all phosphatase activity. I also assessed the effect of protein G129E, which encodes PTEN lacking lipid phosphatase but retains protein phosphatase activity. Lastly, protein Y138L, which encodes PTEN with lipid phosphatase activity but no protein phosphatase activity, was reintroduced into PTEN (-/-) cells (Davidson, 2010).

First, I determined the level of PTEN protein following transfection of the PTEN (-/-) cells with the wild-type vector and compared expression levels with those in the PTEN wild-type PTEN (+/+) cell line (Figure 4.12).

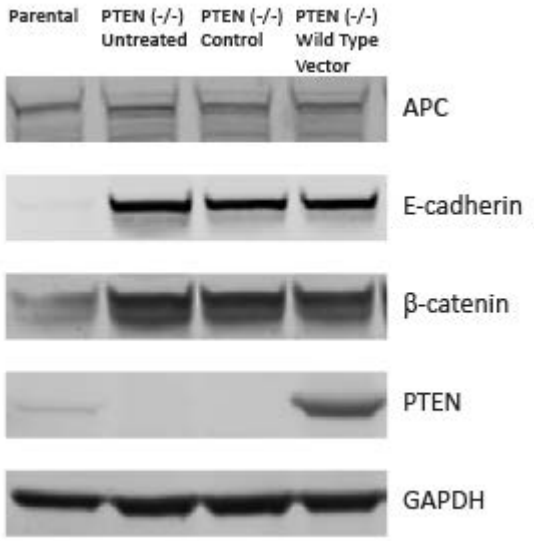



*Figure 4.11: Expression of wild-type PTEN in PTEN (-/-) cells leads to higher levels of PTEN than is present in PTEN (+/+) cells. PTEN (-/-) cells were transfected with either wild-type PTEN or control (empty vector). Whole-cell lysate was subjected to western blot and targeted with antibodies against APC, E-cadherin, β-catenin, PTEN and GAPDH.*

Transfection of wild-type PTEN into PTEN (-/-) cells achieved expression of PTEN protein. The expression level was higher than that of endogenously expressed PTEN in the PTEN (+/+) cell line.

I next measured the effects of the PTEN expression on levels of APC, β-catenin and E-cadherin (Figure 4.13).

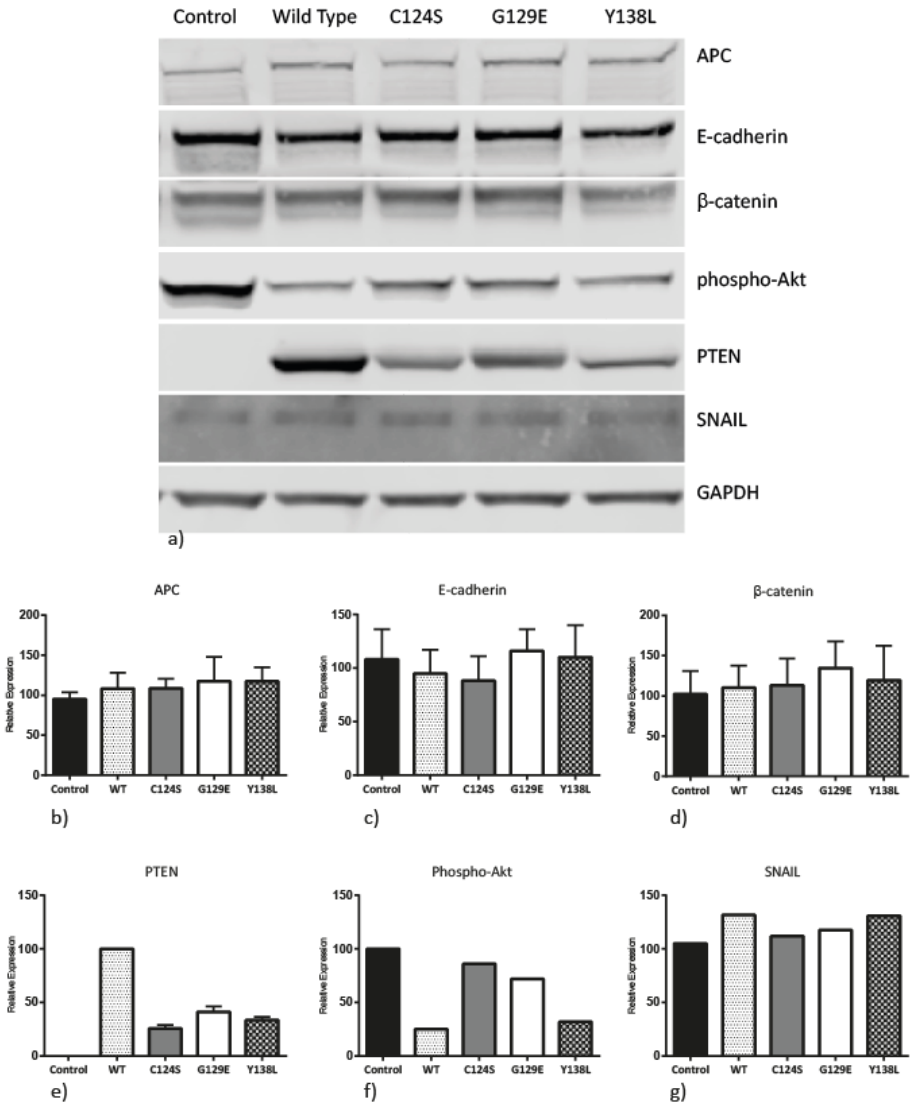



*Figure 4.12: Expression of PTEN protein in PTEN (-/-) cells causes minimal change in the levels of APC, E-cadherin, β-catenin or Snail, while phospho-Akt is responsive. PTEN (-/-) cells were transfected with a panel of PTEN proteins. Whole-cell lysate was probed with antibody against APC, E-cadherin, β-catenin, phospho-Akt, PTEN and Snail (a). Protein levels were quantified relative to GAPDH. Protein levels were plotted relative to levels in the control (empty vector) (b–g). Data represent mean and standard deviation of three independent experiments except those for phospho-Akt and Snail, which represent one replicate.*

PTEN expression in PTEN (-/-) cells causes no change in the protein level of APC, E-cadherin, β-catenin or Snail. Phospho-Akt levels are reduced following expression of PTEN with lipid phosphatase activity (wild type and Y138L).

APC levels are unchanged following transfection of any of the PTEN vectors (Graph (b) Figure 4.14) ($p > 0.05$ for each vector versus control). APC levels in the PTEN (-/-) cells are slightly lower. I expected a fall in β-catenin following transfection of both the wild type and Y138L vectors, which have active lipid phosphatase activity. Re-expression of PTEN with lipid phosphatase activity may have been expected to down regulate Akt and the inhibition of GSK3β. This could potentially result in increased functional efficiency of the APC destruction complex with resultant decrease in β-catenin. This did happen.

Similarly, there was little difference in E-cadherin protein level following expression of any of the PTEN proteins ($p > 0.05$ for each vector versus control).

Expression levels of phospho-Akt are altered as expected in PTEN transfected cells (f). As expected, little change compared with the control was observed following transfection of either C124S or G129E as these both lack lipid phosphatase activity. A reduction to 20% and 30% of p-Akt compared with control levels is observed in cells expressing the wild type and Y138L PTEN, respectively. Both these PTEN proteins retain lipid phosphatase activity and are active in the PTEN/Akt pathway consistent with the decrease in p-Akt in cells expressing these PTEN proteins.

I also measured the effect of different PTEN proteins on the expression of Snail. Snail is implicated in the EMT process. A high level of β-catenin can lead to Snail-mediated repression of E-cadherin transcription (Barrallo-Gimeno, 2005). No change in Snail expression was measured following transfection of any of the PTEN proteins.

To determine if PTEN re-expression altered the transcription of the proteins of interest, I measured the transcription of β-catenin, AXIN2 and E-cadherin in cells transfected with different PTEN proteins (Figure 4.15).

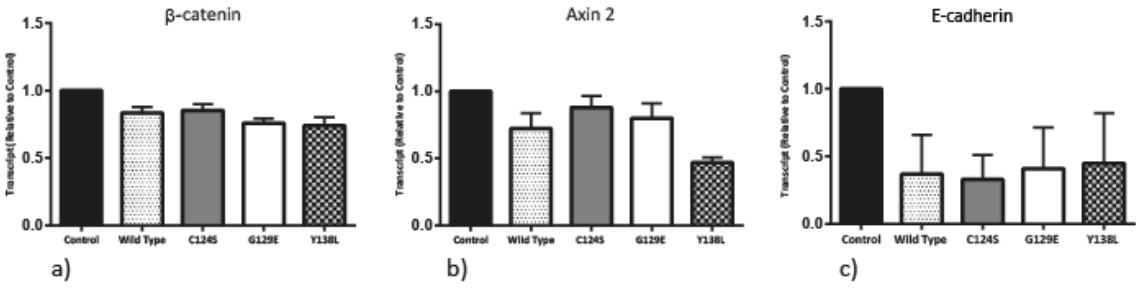



*Figure 4.13: Transfection of PTEN protein into PTEN (-/-) cells causes changes in the transcription of β-catenin, AXIN2 and E-cadherin. PTEN proteins were transfected into PTEN (-/-) cells. Transcript levels of β-catenin (a), AXIN2 (b) and E-cadherin (c) were measured using qPCR. Transcript levels are plotted relative to the control and data reflect the mean and standard deviation of three independent experiments except (c), which represents the mean and standard deviation of seven separate experiments.*

The transcript level of β-catenin (a) is reduced following transfection of all PTEN proteins. AXIN2 transcript is reduced significantly following transfection of PTEN that retains lipid phosphatase activity and E-cadherin transcript is significantly reduced in response to all PTEN proteins.

Transcript level of β-catenin is reduced significantly following transfection of all PTEN proteins. This does not reflect the response of β-catenin protein, which was unchanged under all conditions. The time course of the experiment may have been too short for the transcriptional changes to be translated into changes in protein level. It is surprising that a reduction was observed in response to all proteins as the expectation would have been that a change would have occurred only following transfection of PTEN with lipid phosphatase activity.

E-cadherin transcript was reduced significantly following expression of any of the PTEN proteins. This is an unexpected finding considering that this does not correspond to a reduction in E-cadherin protein. It is possible that the 48 hour time point may not be sufficient for a reduction in transcripts to produce a reduction in E-cadherin protein.

Nonetheless, the reduced E-cadherin transcript is puzzling. All PTEN proteins have this effect, suggesting that this effect is unrelated to either phosphatase activity of PTEN and raising the

question of whether the physical presence of the PTEN protein is somehow involved. Alternatively, the effect of PTEN on E-cadherin may be specific to HCT116 cells and may be related to other genetic defects in these cells. Further analysis is required to clarify this finding.

No significant change in AXIN2 transcription is observed following expression of C124S or G129E. However, Axin transcription is significantly reduced in cells expressing PTEN proteins that have lipid phosphatase activity. Given that changes in β-catenin were not detected under these conditions, it is possible that changes in the availability of β-catenin for transcriptional activation of Axin are responsible. To assess this hypothesis, co-immunoprecipitation of β-catenin could be carried out to assess if its binding with either APC or E-cadherin changed following PTEN transfection in future experiments. Previous experiments showed that a cytoplasmic redistribution of β-catenin was associated with an increase in AXIN2 transcription. This was not necessarily accompanied by an increase in total β-catenin level.

To determine if the PTEN effects I observed are related to the localisation of β-catenin and E-cadherin in cells, I next measured their distribution between different cellular compartments in cells after transfection with different PTEN proteins (Figure 4.16).

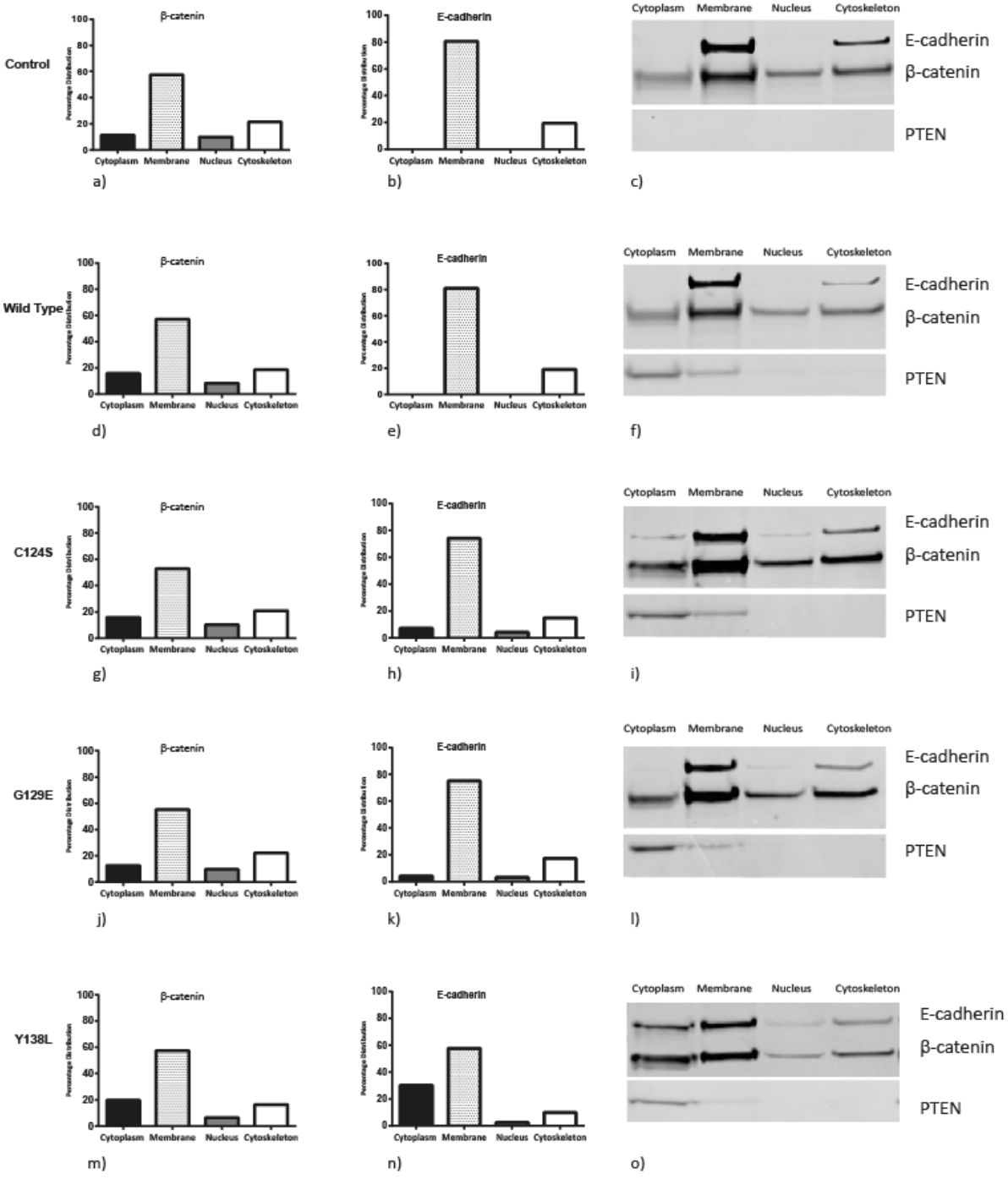



Figure 4.14: *Transfection of PTEN proteins into PTEN (-/-) cells causes no redistribution of β-catenin or E-cadherin. PTEN (-/-) cells were transfected with a panel of PTEN proteins. They were then fractionated into cytoplasmic, membrane, nuclear and cytoskeletal compartments prior to western blot. Membranes were probed with antibodies against β-catenin, E-cadherin and PTEN. The proportional distribution of β-catenin and E-cadherin was calculated and plotted as control (a & b), wild type (d & e), C124S (g & h), G129E (j & k) and Y138L (m & n). Blots are shown in (c, f, i, l and o). The data represent results from one experiment.*

Transfection of all tested PTEN vectors caused no change in the localisation of β-catenin compared with control cells. Similarly, no redistribution was observed for E-cadherin, which was almost exclusively present in the membrane and cytoskeleton fractions. Slightly more E-cadherin was present in the cytoplasmic fraction following expression of PTEN Y138L. This experiment was only performed once and requires repeating prior to drawing firm conclusions.

**Summary of Key Findings:**

- Reintroduction of PTEN into PTEN (-/-) cells caused no change in the level of APC, β-catenin or Snail, while levels of phospho-Akt were significantly reduced following transfection of PTEN with lipid phosphatase activity.
- Transcript levels of β-catenin and E-cadherin were reduced when PTEN protein was reintroduced into PTEN (-/-) cells, while AXIN2 transcript reduced only in response to PTEN protein with active lipid phosphatase activity.

## 4.3 Discussion

In Chapter 4, I have established that PTEN (-/-) cells express higher levels of β-catenin and E-cadherin than (PTEN +/+) cells. The increased level of β-catenin in the PTEN (-/-) cells is likely to be due to a lack of degradation as β-catenin transcription is similar in both cell lines. This could be mediated by activation of the Akt pathway in the absence of PTEN. This in turn can inhibit GSK3β, which could negatively impact on the function of the APC destruction complex. This potentially explains the higher levels of β-catenin. The higher levels of β-catenin protein are reflected by elevated AXIN2 transcript in the PTEN (-/-) cells indicating the β-catenin is transcriptionally active.

The PTEN (-/-) cells were also characterised by higher levels of E-cadherin protein. I initially suspected this was due to stabilisation of the protein by the excess β-catenin. However, I then measured a significantly higher transcript level of E-cadherin in the PTEN (-/-) null cells,

indicating that production of new E-cadherin as opposed to stabilisation is responsible. Currently, the mechanism stimulating this increased transcription is unknown. These findings support those from Chapter 3, where PTEN depletion led to increased levels of E-cadherin protein and transcript in the three PTEN wild-type cells studied. This finding is of relevance to tumours. During the process of EMT as tumours progress and invade, E-cadherin is down regulated. This contrasts with the high E-cadherin levels in these PTEN (-/-) cells, which one may intuitively expect to furnish the cells with less of a migratory phenotype compared with a cell with low E-cadherin. PTEN loss has also been associated with increased cell migration, so the situation may be more complex. Analysis of migration in these cells may clarify this.

The PTEN (+/+) and PTEN (-/-) cells express the same β-catenin. In both cell lines the proportion of β-catenin found in association was similar, which is unsurprising. There was far more β-catenin bound to E-cadherin in the PTEN cells compared with PTEN (+/+). This may be a simple reflection of the fact that there is more β-catenin and E-cadherin in these cells so more interaction occurs. It cannot be determined from these experiments if the mutant protein associates more with APC and the wild type with E-cadherin. Further Co-IP studies of APC and E-cadherin using tagged exogenous β-catenin could be used to investigate this further.

I also measured if the re-introduction of PTEN protein could cause changes in the levels of β-catenin or E-cadherin. No effects were measured on the level of either β-catenin or E-cadherin protein. This contrasted with predictions as I expected PTEN with intact lipid phosphatase activity to cause a reduction in β-catenin. The lipid phosphatase active PTEN proteins did cause a significant decrease in levels of Akt, as predicted.

The transcript level of β-catenin and E-cadherin both decreased in response to all of the PTEN vectors. Although reductions in both were statistically significant, E-cadherin transcript showed the most dramatic response. For both transcripts these results were surprising. These findings support the observation that E-cadherin transcript increases following PTEN depletion of the

PTEN (+/+) cells and in the examined cell lines in Chapter 3. The fact that transcriptional repression followed transfection of all of the PTEN proteins suggests that the physical presence of PTEN is important as opposed to a specific phosphatase function of PTEN. The exact mechanism here is unclear from these experiments.

I have also established that absence of PTEN does not alter the cells' response to APC depletion. In both the PTEN (+/+) and PTEN (-/-) cells, APC knockdown caused a redistribution of β-catenin to the cell cytoplasm with an associated increase in AXIN2 transcription. AXIN2 transcription is already elevated in PTEN (-/-) cells, presumably secondary to elevated β-catenin. APC depletion further amplifies this. These data highlight how the accumulation of mutations in a tumour has the potential to influence cell behaviour and tumour progression. The elevated β-catenin transcriptional output following loss of either APC or PTEN may not be sufficient to cause a major insult to the cell. Loss of both, however, augments this transcriptional activity of β-catenin and could potentially create an environment that predisposes the cell to a more malignant phenotype.

# 5 Functional Consequences of Stabilising β-Catenin Mutations in HCT116 Cells

## 5.1 Introduction

In the preceding chapters, I have established that manipulation of β-catenin, PTEN or APC can cause changes in the relative interactions of β-catenin with either APC or E-cadherin. Dysfunction of this triad of proteins is centrally implicated in the pathogenesis of colorectal cancer. Specifically, in Chapter 3, I established that a point mutation causing stabilisation of β-catenin causes it to associate more with APC but much less with E-cadherin than wild-type β-catenin. In contrast, I identified that absence of PTEN is associated with a higher level of E-cadherin and more association of β-catenin with E-cadherin in HCT116 colorectal cancer cells. All cells studied, irrespective of the presence of stabilised β-catenin or PTEN, responded in a similar fashion to depletion of APC. In all cells, this resulted in a redistribution of β-catenin from the plasma membrane to the cytoplasm and was accompanied by a significant increase in transcriptional output of β-catenin measured by an increase in AXIN2 transcription.

These findings indicate that the contribution β-catenin makes to its roles in cell adhesion or transcription can be affected by defects implicated in cancer. However, these molecular findings have more relevance if they can be translated into effects on cell function. Aberrations in cell function or behaviour is key to driving cancer progression.

Gut epithelial cells have several functions important in maintaining homeostasis. Stem cells at the crypt base must undergo controlled proliferation to produce the specialised cell types that populate the crypt (Bjerknes and Cheng 2006, Leushacke and Barker 2012). The differentiated cells then migrate in an orderly way up the crypt towards the gut lumen. Cell migration along the crypt ends when cells either apoptose or undergo anoikis and are shed into the gut lumen. From birth of a cell to its loss takes approximately three days (van der Flier, 2009). This rapid

turnover of cells should ensure that cells with oncogenic mutations are usually quickly lost from the tissue, minimising chances of acquiring further defects. Following tumour development, it can metastasise to distant organs. To do this it must be able to invade surrounding tissues.

In this chapter, I will measure the effects of mutations in β-catenin and PTEN as well as loss of APC on cell function and morphology.

Specifically, I will:

1) Determine if mutations in β-catenin or PTEN affect cell size and proliferation.
2) Determine if mutations in β-catenin or PTEN as well as depletion of APC can cause changes in cell migration and invasion.

## 5.2 Results

### 5.2.1 Stabilising point mutation of β-catenin reduces cell size

Cell size and morphology in normal epithelial tissue is uniform and consistent. This uniformity can be lost in tumours with cell shape and size being more variable. To measure the impact of stabilising β-catenin mutations on cell size, I used fluorescence-activated cell sorting (FACS) to measure cell size based on forward scatter. This provided a basic morphological characterisation of the cells and determined how this was influenced by stabilising β-catenin mutations or depletion of APC, β-catenin or PTEN (Figure 5.1).

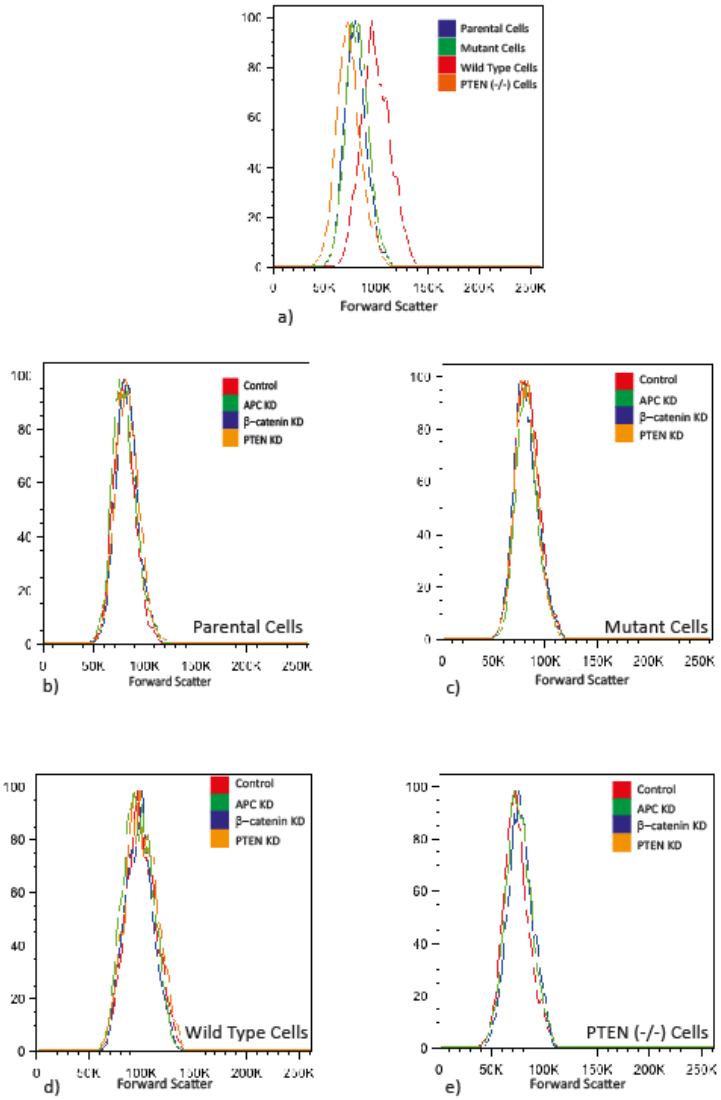



*Figure 5.1: Cells expressing stabilised β-catenin are smaller than those expressing only wild-type β-catenin. Cells were treated as control (scrambled siRNA) or had APC, β-catenin or E-cadherin depleted prior to analysis of cell size using FACS based on forward scatter. Plots indicate forward scatter as compared between cell lines under control conditions (a) or following depletion of proteins outlined in the parental (b), mutant (c), wild type (d) and PTEN (-/-) (e). Plotted data are representative. The experiment was performed on at least three separate occasions.*

Cells expressing mutant β-catenin were consistently smaller compared with cells expressing only wild-type β-catenin (i.e. no stabilised β-catenin). Depletion of APC, β-catenin or PTEN did not affect cell size in any of the cells studied.

Cell size has been linked to cell cycle progression and rates of proliferation, which co-operate to proportion organs at the appropriate size (Ginzberg, 2015, Su, 1998). Studies in *Drosophila* have

shown that genetic manipulation of mitosis resulting in an inhibition of proliferation leads to an increase in the size of cells (Weigmann, 1997). Similarly, cell cycle arrest in *Drosophila* results in an increase in cell size whereas increased expression of cell cycle regulators increases proliferation rate with an accompanying decrease in cell size (Neufeld, 1998). Results here may be related to differences in proliferation rates.

### 5.2.1 Summary of Key Finding:

- Cells containing a mutant β-catenin protein are smaller than cells with only wild-type β-catenin protein.

### 5.2.2 Stabilising Mutation in β-Catenin and PTEN Loss Increase the Proliferation Rate of HCT116 Cells

I next aimed to determine the influence of stabilised β-catenin on the rate of cell proliferation.

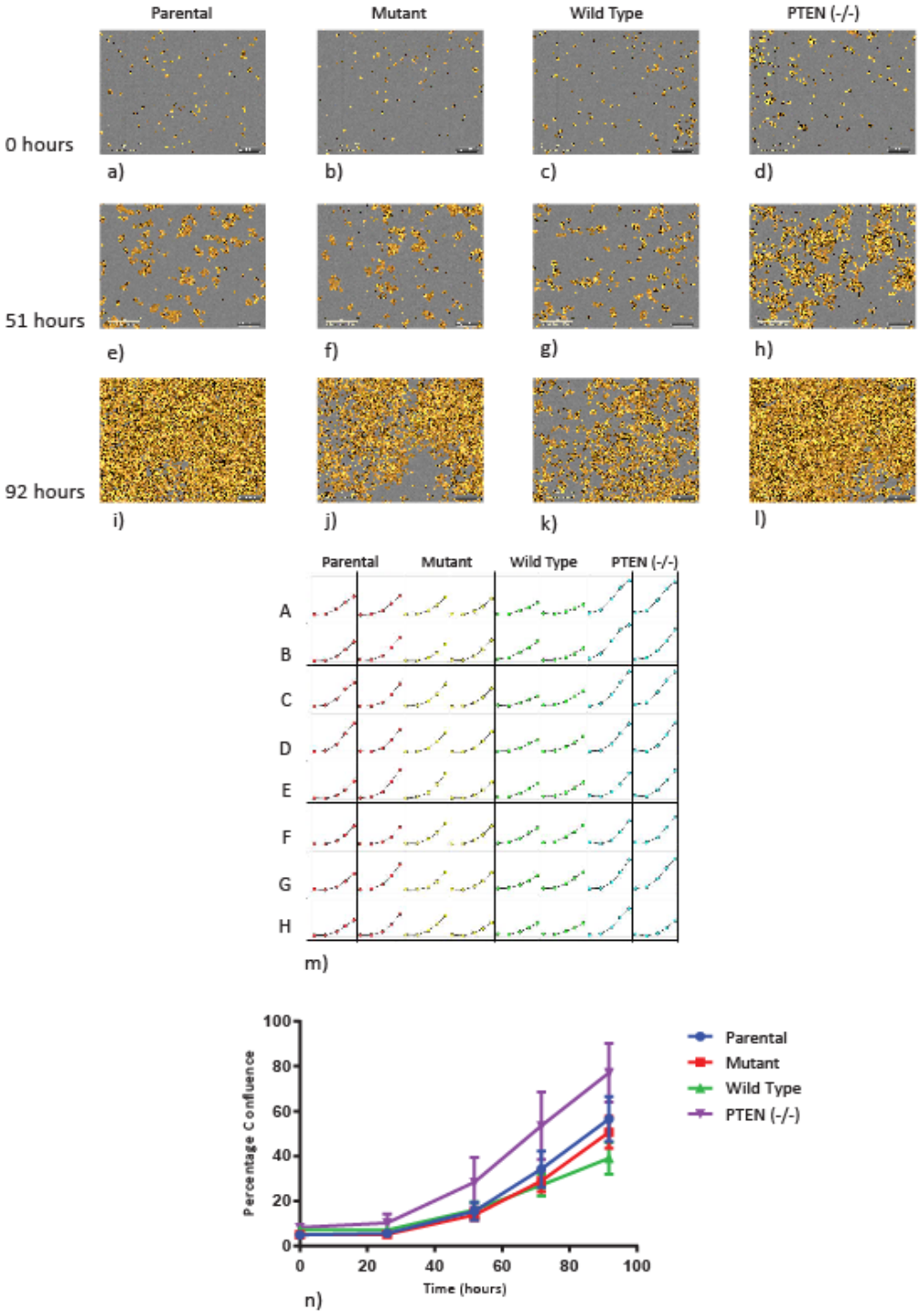



*Figure 5.2: Proliferation is fastest in PTEN (-/-) followed by parental, mutant and wild-type cells. Cells were seeded at approximately 10% confluence in a 96-well plate and incubated in the incucyte zoom system. Each well was imaged every 24 hours for 96 hours and the surface covered by cells calculated by the incucyte software. Confluence shown at 0 (a–d), 51 (e–f) and 92 hours is highlighted in gold and was used to calculate percentage confluence. Consistency of replicates between wells is plotted in (m) showing real time percentage confluence for the duration of the experiment for each well analysed. The mean and standard deviation of percentage confluence from 16 separate wells is plotted in (n).*

PTEN (-/-) cells proliferated at the fastest rate followed by the parental, mutant and wild-type cells. In addition to expressing both stabilised and wild-type β-catenin, the PTEN (-/-) cells may have a proliferative advantage due to activation of the Akt pathway. The order of proliferation rate in the other cell lines followed the pattern of β-catenin expression. The parental cells with one mutant and one wild-type β-catenin were fastest followed by the mutant cells, with only mutant non-degradable β-catenin and lastly the wild-type cells, which only express wild-type β-catenin. The differences were statistically significant between the PTEN (-/-) cells and the other cell lines ($p<0.05$). The parental and mutant cells proliferated significantly faster than the wild type ($p<0.05$) but there was no difference between them ($p>0.05$).

These data show that stabilising mutations in β-catenin provide a proliferative advantage. The stabilisation of β-catenin in these cells is caused by a mutation that renders it non-degradable. This is mechanistically slightly different to the scenario in APC-deficient cells in colorectal cancer. The outcome, i.e. increased proliferation, appears to be similar.

### 5.2.2 Summary of Key Findings:

- Cells expressing mutant β-catenin protein proliferate faster than those with only wild-type β-catenin
- PTEN (-/-) cells proliferate faster than PTEN (+/+) cells

#### 5.2.3.1 Cells with stabilising mutations in β-catenin migrate faster than cells expressing only wild-type β-catenin

Cell migration is vital for normal function of the gut epithelium. Cells migrate upwards in the crypt prior to extrusion into the gut lumen. To occur normally, cells must migrate at the appropriate rate and in the correct direction. Experimental elimination of mitosis does not

prevent the upward translocation of cells, indicating that migration is an active process and can be uncoupled from proliferation {Kaur, 1986). Migration defects could potentially predispose to both the initiation and progression of cancer.

I measured how stabilising mutations in β-catenin affected the migration rate of HCT116 cells into an artificially generated 'wound' (Figure 5.3).

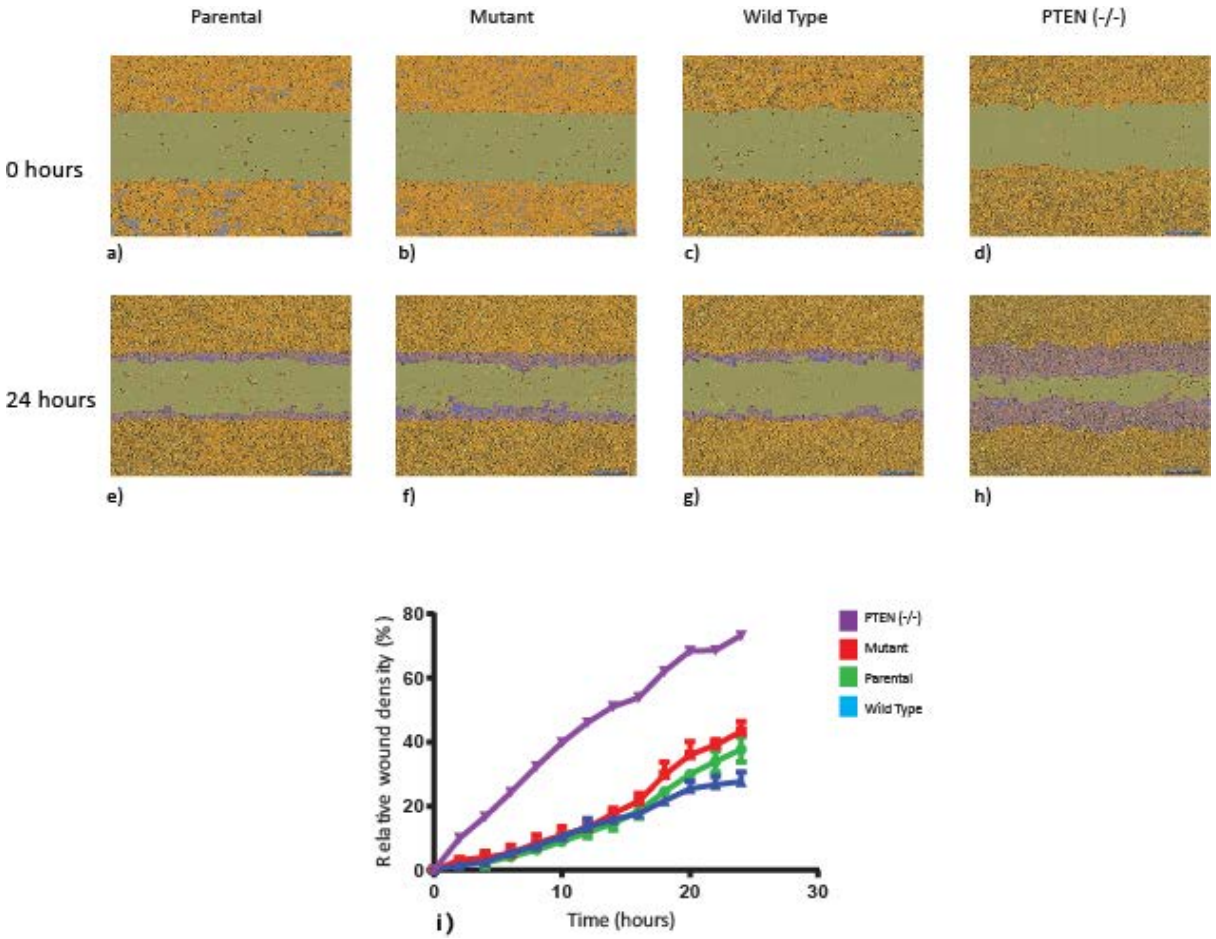



*Figure 5.3: HCT116 cells expressing stabilised β-catenin migrate faster than those expressing only wild-type β-catenin and PTEN (-/-) cells migrate faster than PTEN wild-type cells. Confluent cell monolayers were 'scratched' to create an empty space and migration of cells into the space was measured. Images of the scratch wound are shown at 0 hours (a–d) and 24 hours (e–h) for each cell line. Migration rate is quantified based on the relative area of the wound covered by cells. The gold overlay represents the 'mask' that marks the surface covered by cells and is used to quantify relative wound space. Purple shading shows the area of the wound covered in 24 hours. Relative wound space covered is plotted against time in (i). Each data point represents the mean and standard deviation of six replicate wells.*

PTEN (-/-) cells migrated significantly faster ($p<0.05$), followed by the mutant parental then wild-type cells. All cell lines expressing stabilised β-catenin migrated significantly faster than cells containing only wild-type β-catenin. The PTEN (-/-) cells have the same complement of β-catenin as the parental cell line (Δ45/+). The absence of PTEN augmented the rate of migration, which is consistent with previous findings (Raftopoulou, 2004, Leslie, 2007, Davidson, 2010). Cells expressing stabilised β-catenin also proliferated faster than cells with only wild-type β-catenin. A degree of proliferation at the wound edge may contribute to the distance the cells moved into the wound. Limiting the experiment to 24 hours sought to minimise this effect.

#### 5.2.3.2 APC depletion slows migration irrespective of β-catenin

APC loss is an early and common event in colorectal cancer. It serves an important function in the regulation of β-catenin but also has multiple other roles, including those related to microtubules and cell migration (Näthke, 1996, Mimori-Kiyosue, 2000, Nelson, 2013). Previous studies have suggested that APC loss may negatively affect cell migration (Nelson, 2012). I aimed to determine if APC depletion impacted on the migration of HCT116 cells and if any measured effects were dependent on the expression pattern of β-catenin. In addition, I measured the effect of depleting β-catenin or PTEN on cell migration (Figure 5.4).

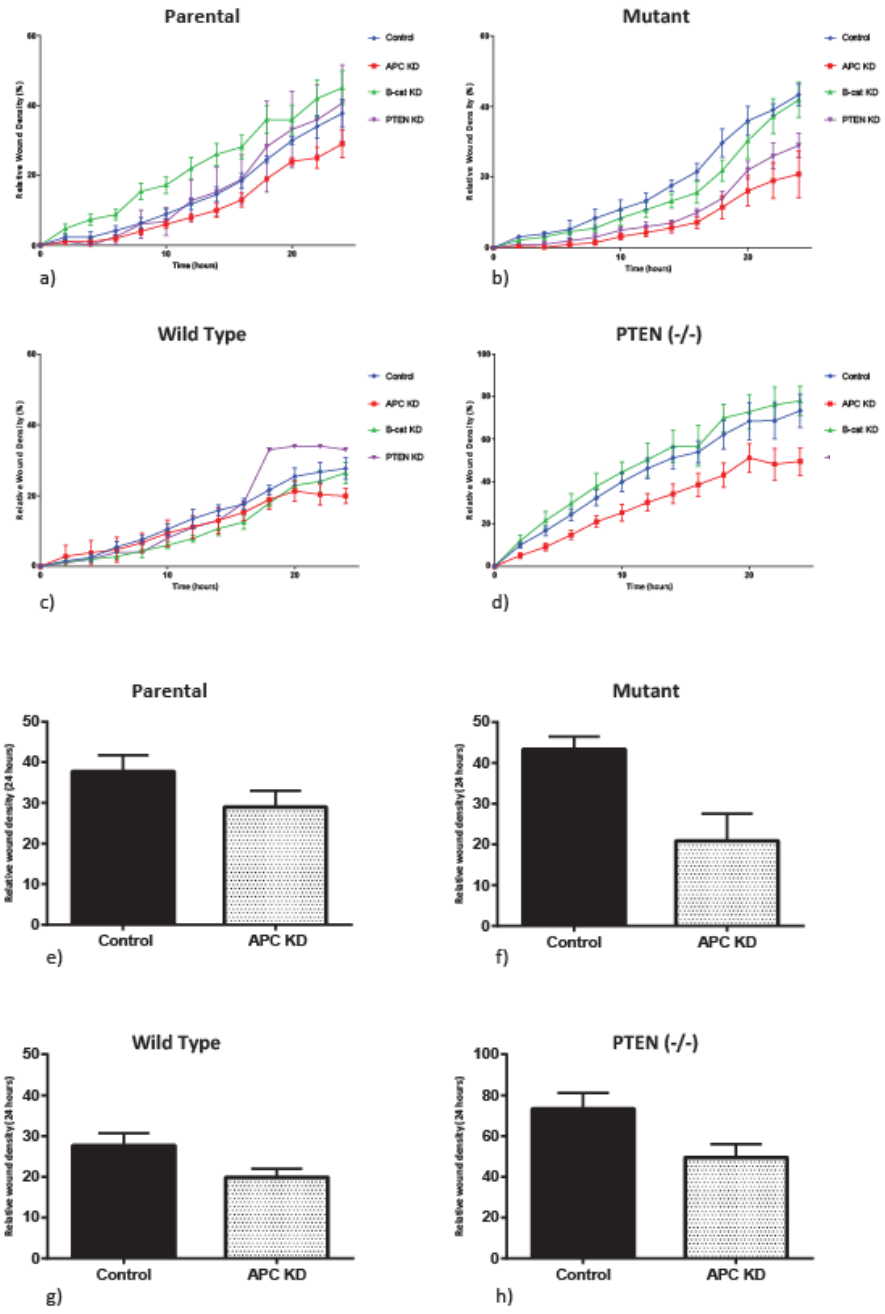



*Figure 5.4: APC depletion causes a reduction in migration rate irrespective of β-catenin status. Cells were depleted of APC, β-catenin or PTEN. A scrambled siRNA was used as a control. Cells were then plated in a 96-well plate and allowed to reach confluence overnight. The confluent monolayer of cells was scratched and incubated in the incucyte zoom system. Images of the cells were captured every 2 hours for 24 hours. The area of the wound covered by the migrating edge of the cells was used to calculate migration rate. Relative wound confluence for parental (a), mutant (b), wild-type (c) and PTEN (-/-) cells (d) is plotted against time. Comparison between relative wound confluence at 24 hours in the control versus APC knockdown conditions are plotted in (e–f). Data presented are representative of two independent experiments with six replicates in each for each treatment condition.*

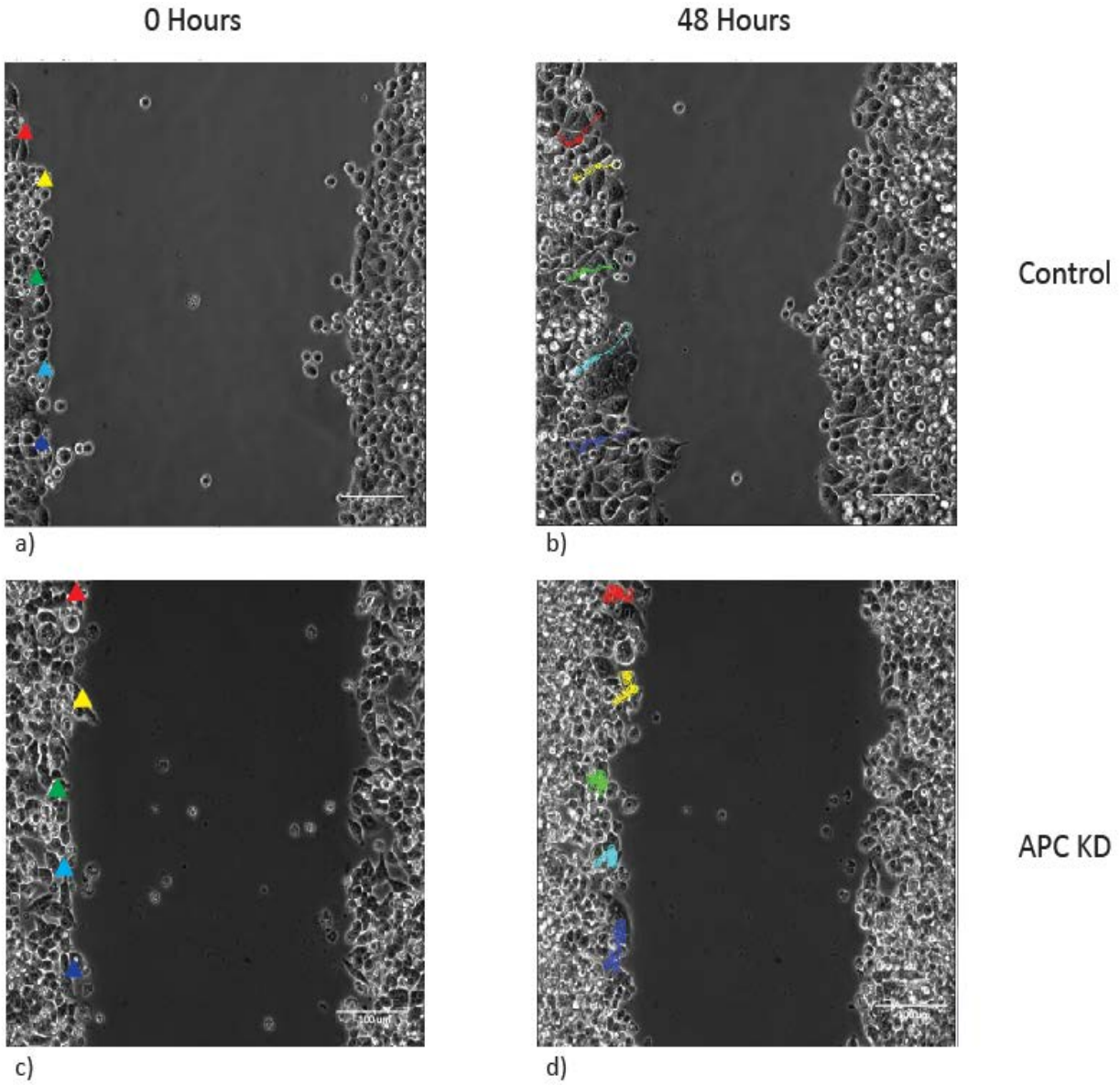
APC depletion reduces migration of HCT116 cells independently of β-catenin status. The rate of migration was less in all cell lines following APC knockdown. This reached statistical significance in all cell lines ($p < 0.005$).

Depletion of PTEN did not cause an increase in migration rate, which may have been expected given that PTEN null cells migrated faster than PTEN wild-type cells. Although siRNA directed towards PTEN achieved a good knockdown on western blotting, the knockdown may have been heterogeneous in the migration assay. Cells with incomplete knockdown may migrate at a slower rate than cells that are completely devoid of PTEN. Cells were treated for 48 hours with siRNA prior to commencement of the migration assay. Perhaps changes required to increase migration speed in response to PTEN absence require a longer time period to produce a detectable phenotype.

There are two possible effects that could account for the reduction in migration in APC-depleted cells. Either reduced migration speed, or a loss in directionality. I aimed to distinguish between these possibilities

#### 5.2.3.3 APC depletion reduces total distance migrated by a cell but also causes loss of directionality. This occurs independently of β-catenin

To assess if reduced migration following APC depletion was secondary to a reduction in total distance travelled by the cell or loss of directionality, I tracked cells migrating across a wound. In this section, I compare cells expressing only stabilised β-catenin (mutant cells) and those expressing only wild-type β-catenin (wild-type cells). Cells were tracked for 48 hours while migrating across a wound. The total distance travelled between the start and end point was measured. In addition, the distance separating the position of the start and end point was also noted to determine the net displacement over 48 hours (Figure 5.5).


0 Hours
48 Hours
Control
APC KD
a)
b)
c)
d)

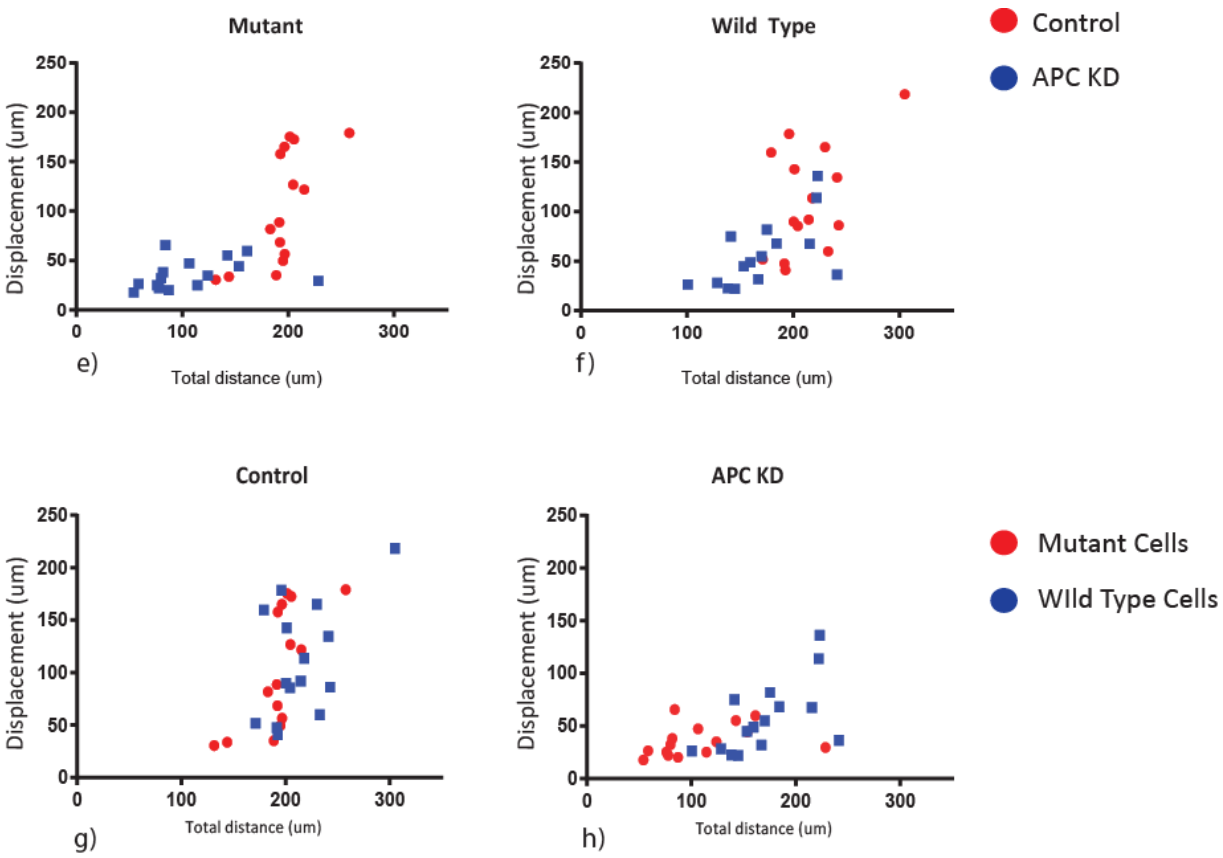



*Figure 5.5: APC depletion causes a reduction in total distance travelled and also in the degree of displacement, indicating a loss of direction. Cells were tracked migrating across a wound for 48 hours. The total distance travelled was calculated in addition to the displacement from the start point at 48 hours. A representative image shows the start location and migration path for five separate cells in the control versus APC knockdown conditions (a–d). Total migration distance is plotted against displacement for 15 separate mutant (e) and wild-type (f) cells to compare the migration pattern in the control versus APC-deplete conditions. Red represents the control and blue the APC-deplete conditions. A comparison between migration patterns in mutant versus wild-type cells under control (g) and APC-deplete (h) conditions was made. Red represents mutant and blue the wild-type cells.*

In both cell lines, APC depletion causes a reduction in total migration distance and in displacement. This indicates that the ability to migrate is negatively affected following APC loss, but in addition the cells do not know where they are going. Depletion of APC had similar effects on migration in both the mutant and wild-type cells. These data indicate that the effects of APC depletion on cell migration occur independently of whether mutant or wild-type β-catenin is expressed. Loss of directional migration is represented schematically (Figure 5.6).

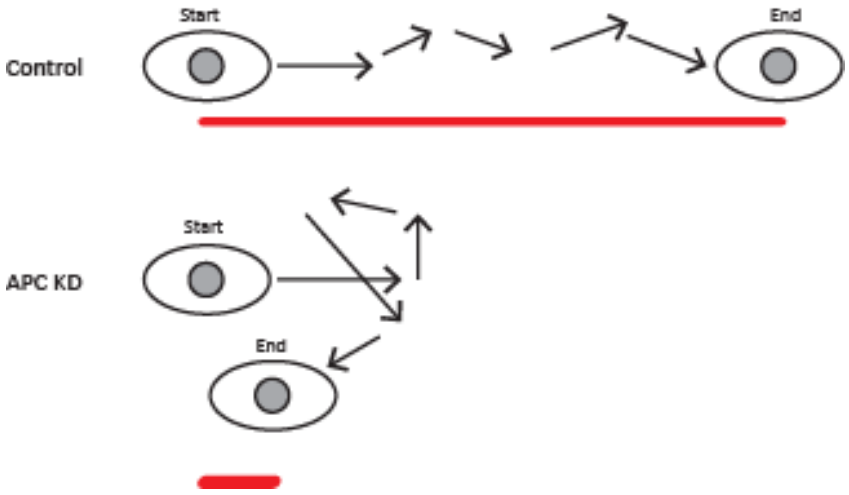



*Figure 5.6: A schematic representation of the effects of APC depletion on cell migration. The upper cells represent the control and the lower cells APC-deplete conditions. Total distance travelled is shown by the black arrows. The distance from the start to finish point is shown by the red lines.*

### 5.2.3 Summary of Key Findings:

- Cells expressing mutant β-catenin migrate faster than those with only wild-type β-catenin.
- PTEN (-/-) cells migrate faster that PTEN (+/+) cells.
- APC depletion reduces rate of migration in all cell lines and this is due mainly to loss of directionality.

### 5.2.4 Cells with stabilising mutations in β-catenin are less invasive than cells with only wild-type β-catenin

Invasion is an important step in the progression of cancer and advanced invasive lesions are associated with a worse outcome than earlier lesions. I assessed whether stabilising mutations in β-catenin influence this process by measuring the invasion of HCT116 cells and I also compared the effect of depletion of APC, β-catenin or PTEN.

I again used the incucyte zoom imaging system (Essen Bioscience). A scratch wound assay is set up, then a layer of matrigel is placed on top of the wound and allowed to set. Growth medium is then placed on top and the assay is allowed to run as a normal migration experiment. The matrigel barrier means that to migrate into an empty space cells invade through the matrigel. A schematic of this experimental set up is shown (Figure 5.7).

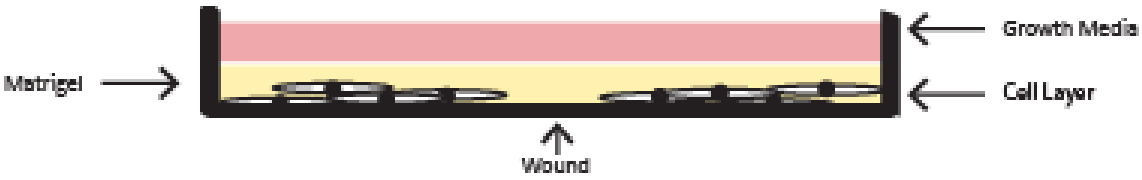



*Figure 5.8: Schematic of invasion assay set-up. A wound is made in a cell monolayer. Matrigel is placed on top, allowed to set, and then growth medium is placed on top. The matrigel provides a physical barrier for the cells to invade.*

Data showing comparisons between cell lines under control conditions are shown (Figure 5.9).

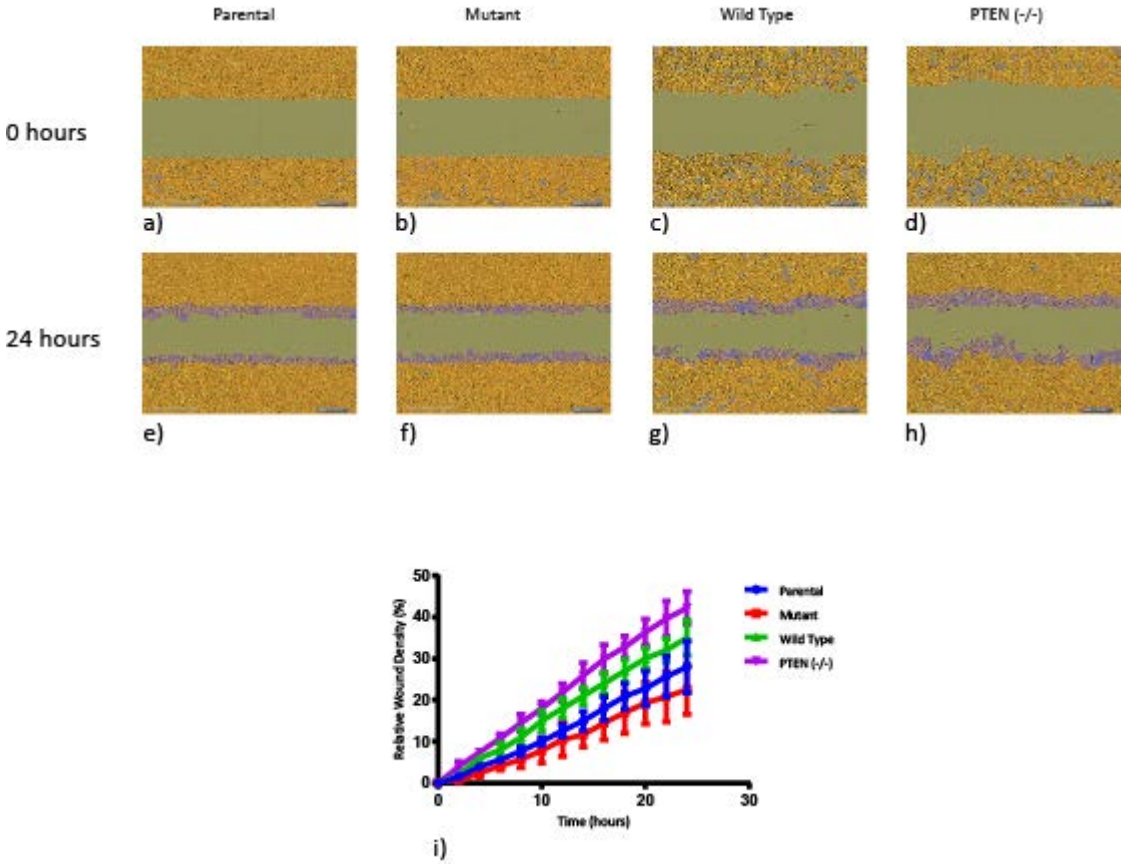



*Figure 5.9: PTEN (-/-) cells invade through a matrigel barrier faster than PTEN (+/+) cells and cells expressing only wild-type β-catenin invade faster than cells with stabilised β-catenin. A confluent monolayer of cells was scratched and a layer of matrigel placed on top and allowed to set. Cells were then incubated in the incucyte zoom system. Images of the cells were captured every two hours for 24 hours. Relative wound density is plotted against time in (i). Each data point represents the mean and standard deviation of six replicate wells.*

PTEN (-/-) null cells invade faster than PTEN wild-type cells ($p$ <0.05). Cells expressing only wild-type β-catenin invade faster than cells with stabilised β-catenin. Differences in relative wound density at 24 hours were significant when comparing parental ($p$ <0.05) and mutant ($p$ <0.05) with wild type.

The findings in the PTEN (-/-) cells reflect their high rate of cell migration. The invasion patterns of the other three cell lines are the opposite of their order of migration speeds, where mutant was fastest over parental then wild-type cells. The reasons for this could be multiple as migration and invasion are two different processes.

I next measured the effects of depleting APC, β-catenin and PTEN on invasion rate (Figure 5.10).

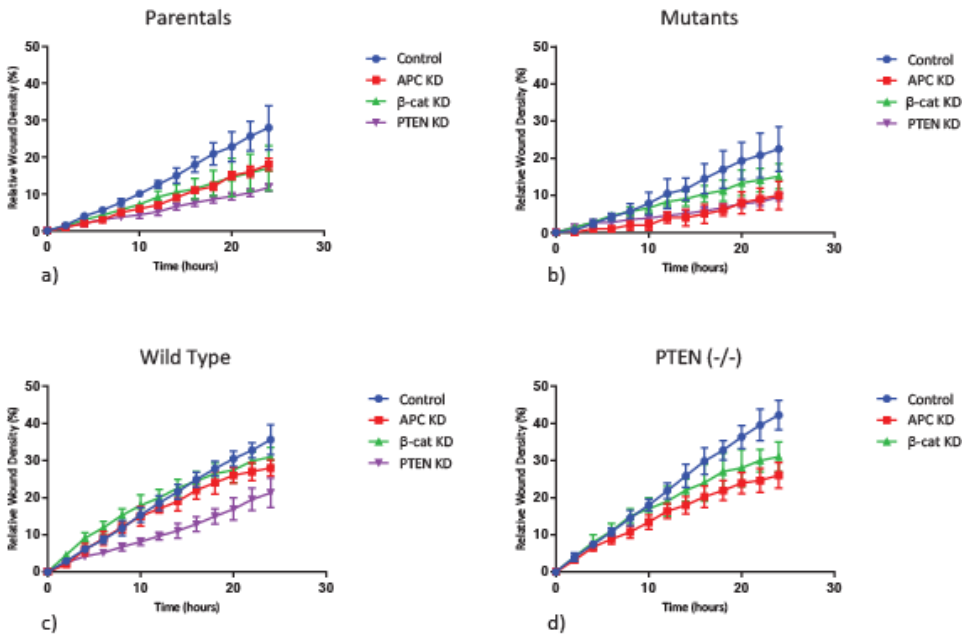



*Figure 5.10: Depletion of β-catenin, APC or PTEN slows invasion rate in all cell lines. APC, β-catenin or PTEN were depleted from cells. Control cells were targeted with scrambled siRNA. A confluent monolayer of the cells was scratched and a layer of matrigel placed on top. Cells were then incubated in the incucyte zoom system. Images were captured every two hours for 24 hours. Invasion rate was quantified based on relative wound density (or area covered by cells). Change in wound density over 24 hours is plotted for parental (a), mutant (b), wild-type (c) and PTEN (-/-) cells (d). Each data point represents the mean and standard deviation from six replicate wells.*

Depletion of APC, β-catenin or PTEN in all four cell lines caused a reduction in invasion. The greatest and most consistent effects were detected following APC or PTEN knockdown.

Invasion at 24 hours was significantly lower in all four cell lines following APC depletion compared with the control ($p < 0.005$ for all cells compared with the control). The reduction in invasion following APC depletion mirrors the reduced cell migration rate. Depletion of β-catenin and PTEN also slowed the rate of invasion.

I feel the invasion data should be interpreted with caution. Although two repeats of the experiment were performed each with six wells per cell line, the cells were “unhappy” in the matrigel. This is an anecdotal observation and I have no quantitative data pertaining to this.

Even during trial growth periods in matrigel in preparation for the invasion the cells did not seem to enjoy being grown under these conditions. I think there is a possibility that some of the data may be skewed by growth conditions of the cells.

#### 5.2.4 Summary of Key Findings:

- PTEN (-/-) cells invade at a faster rate than PTEN wild-type cells.
- Cells with only wild-type β-catenin protein invade faster than cells expressing mutant β-catenin.
- APC depletion reduces invasion in all cell lines studied.

## 5.3 Discussion

In this chapter, I aimed to relate some of the changes I discovered at the molecular level in Chapters 3 and 4 to cell function and phenotype. I have established that cells expressing mutant β-catenin proliferate faster than cells expressing only wild-type β-catenin. Intuitively this may be expected. However, in Chapter 3 I showed that β-catenin transcriptional output measured by AXIN2 transcription was not significantly different between the cell lines. This may indicate that other β-catenin target genes are responsible for the observed effects on proliferation. The wild-type cell line had the lowest rate of proliferation. I also showed that the wild-type cells were larger than cells expressing mutant β-catenin. The proliferation assay measures area covered by the cells as an indicator of proliferation. The rate in the wild-type cells may therefore have been exaggerated by their greater size. Differences in cell size may in fact be due to differences in the rate of proliferation. Cells lacking PTEN proliferated fastest of all. Their proliferation rate is likely to be augmented by activation of the Akt pathway.

PTEN (-/-) cells also migrated fastest. Loss of PTEN has been linked to increased migration previously and is related to absence of its protein phosphatase activity. Cells expressing mutant β-catenin migrated faster than those with only wild-type β-catenin. Some degree of proliferation

at the migrating cell edge may have contributed to this, but the experiment was limited to 24 hours, minimising effects. A more rapid cell migration in the mutant cells may be aided by low expression levels of E-cadherin. Down regulation of E-cadherin is known to contribute to the migratory phenotype associated with the process of EMT. The increased migration rate in cells with mutant β-catenin could potentially impact on tumour development. Ordered directional migration up the colonic crypt prior to shedding of cells into the lumen is required for gut homeostasis. A cell with a stabilising mutation in β-catenin may migrate up the crypt faster than normal cells. This could minimise the time the cell has to acquire further mutations, reducing the chance of tumour development.

I also determined that APC depletion reduces cell migration significantly irrespective of whether mutant or wild-type β-catenin is expressed. This finding indicates that the observed effects of APC depletion occur independently of β-catenin. Two aspects of cell migration were affected by APC loss. The total distance travelled by the cell was reduced but the displacement of the cell over the time course of the experiment was also significantly less. This indicates that the cells have lost their sense of direction and they do not know where they are going. In all cell lines APC depletion caused a redistribution of β-catenin to the cytoplasm with only modest increases in total cell levels. This was also accompanied by an increase in AXIN2 transcription. I hypothesise that the migratory defects are not, however, related to the regulation of β-catenin. Observed effects may be due to loss of APC from its role related to microtubules and cell movement. This hypothesis could be tested in several ways. Depolymerisation of microtubules may be expected to produce similar results to APC loss. Measurement of migration could also be undertaken following overexpression of β-catenin. If my hypothesis that migratory defects following APC loss are unrelated to β-catenin is correct, then overexpression would not be expected to impact on migration.

APC depletion and stabilising mutations of β-catenin have opposite effects on cell migration. These effects may be the result of distinct mechanisms. β-Catenin mutations could result in an increased migration rate due to low expression levels of E-cadherin. In addition, transcriptional activity of the stabilised β-catenin could also have effects on genes other than AXIN2 that influence migration. APC loss, on the other hand, may negatively impact cell migration due to loss of function in its role associated with microtubules. Migratory defects have relevance to colorectal tumours. If APC loss results in a cell losing its sense of direction, instead of the cell being expelled into the intestinal lumen, its residence in the crypt may be prolonged. This could provide an opportunity for the cell to acquire further mutations necessary for tumour progression. The functional read out is different from that resulting from stabilising β-catenin mutations, which increase migration rate. Increased expulsion of a cell from the crypt that retains a sense of direction may reduce the risk of the cell acquiring further defects.

# 6 β-Catenin Localisation in Gut Organoids and Human Intestinal Polyps

## 6.1 Introduction

I have established that stabilising β-catenin mutations influences its interaction with APC and E-cadherin, and that PTEN depletion is associated with increased E-cadherin expression. APC depletion in all cell lines studied caused a redistribution of β-catenin from the plasma membrane to the cytoplasm and was associated with an increase in AXIN2 transcription. APC depletion also caused defective cell migration.

I wanted to establish if previous findings in cells could be repeated in a more physiologically relevant system by using gut organoids. I also aimed to assess if β-catenin was retained at the plasma membrane or enriched in the nucleus of human colorectal polyps. PTEN loss has been implicated in the serrated pathway of colorectal carcinogenesis. I intended to establish if serrated polyps also expressed higher levels of E-cadherin that characterised HCT116 cells following PTEN depletion.

Organoids are self-organising three-dimensional epithelial structures. These “mini guts” can be cultured and grown *in vitro*. They contain stem cells, Paneth cells and all the specialised differentiated cell lineages that populate the *in vivo* crypt–villous structure. They provide an experimental system that is physiologically closer to *in vivo* gut tissue than cultured cell lines.

Organoids initially form rounded structures, which then form buds that elongate to form the crypt unit. Stem cells resident at the crypt base give rise to all specialised types of terminally differentiated epithelial cells, which migrate up the crypt and are shed into the central lumen of the organoid. The morphology of organoids derived from APC $^{Min/+}$ mouse gut differs from the branched wild-type structure. APC $^{Min/+}$ organoids form round cyst-like structures upon loss of the second *Apc* allele. This phenotypic change is thought to result from overstimulation of the Wnt pathway following APC loss. A similar cyst structure is characteristic of organoids derived

from APC $^{fl/fl}$ tissue (Figure 6.1). Assessment of both wild-type and APC $^{Min/+}$ organoids provides the opportunity to measure the influence of different levels of Wnt pathway activation on the localisation of β-catenin.

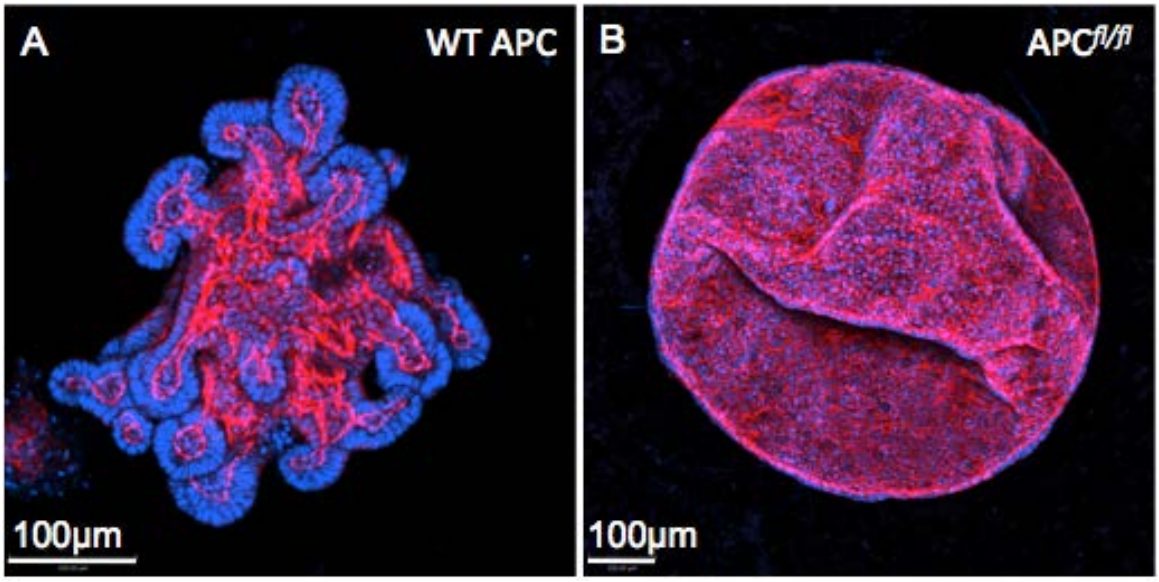



*Figure 6.1: The characteristic branching structure of a wild-type organoid is shown in (A). The typical phenotypic change following APC loss (B) shows loss of architecture and the development of a cyst. Organoids are stained with phalloidin (red) to visualise F-actin and DAPI (blue) to visualise nuclei. (Image courtesy of Dr Paul Appleton).*

## 6.2 Results

### 6.2.1 Localisation of β-catenin in wild-type and APC Min organoids

I measured total levels of APC and β-catenin in wild-type organoids and made comparison to transformed cyst organoids derived from APC $^{min/+}$ tissue. I then measured the distribution of β-catenin in wild-type organoids in comparison to organoids derived from APC $^{Min/+}$ mice. The organoids derived from APC $^{Min/+}$ tissue in this experiment had already transformed into cysts at the time of lysis. This phenotypic change is thought to coincide with loss of APC heterozygosity (Figure 6.2).

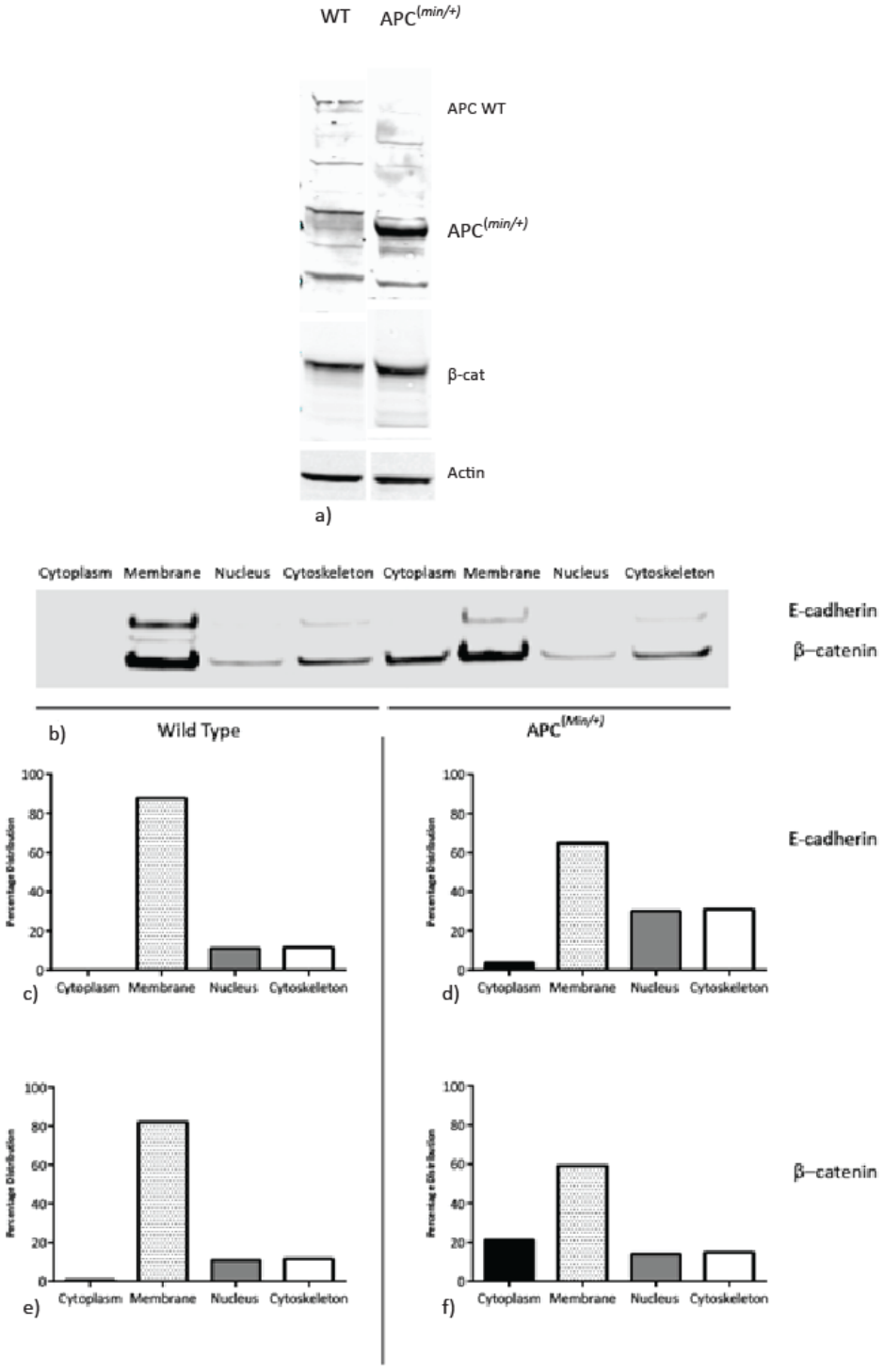



*Figure 6.2: β-Catenin in wild-type organoids is located at the plasma membrane and expressed at a lower level than β-catenin from APC $^{min/+}$ organoids. Organoids lysates were prepped whole or fractionated into cytoplasmic, membrane, nuclear and cytoskeletal fractions. Lysates were subjected to western blotting. Proteins were detected with antibodies against APC, β-catenin, E-cadherin and actin. Localisation of E-cadherin (c & d) and β-catenin (e & f) is plotted as a percentage of cell total. For this experiment, n = 1. Organoids and the whole-lysate western blot were provided by Dr Alistair Langlands.*

Transformed organoids from APC $^{min/+}$ tissue have higher levels of β-catenin than wild-type organoids. β-Catenin localises predominantly at the membrane in wild-type organoids, as does E-cadherin. In the APC $^{min/+}$ organoids, there is more cytoplasmic β-catenin, although the membrane remains the main site of localisation.

Loss of APC in the organoid eliminates the ability to degrade β-catenin, consistent with the higher expression levels of β-catenin in the APC $^{min/+}$ organoid. This supports the elevated β-catenin levels following APC depletion in the parental and wild-type cells in Chapter 3.

APC depletion led to a redistribution of β-catenin from the plasma membrane to the cytoplasm in all cell lines studied in Chapter 3. These findings are reaffirmed in the APC $^{min/+}$ organoid, which has a greater proportion of cytoplasmic β-catenin than the wild type, where the cytoplasm is virtually devoid of β-catenin. Measurement of AXIN2 transcription would be an additional experiment here and I suspect the APC $^{min/+}$ organoids would have a higher transcript level.

E-cadherin was predominantly located at the cell membrane, as expected in both the wild-type and APC $^{min/+}$ organoids.

**6.2.1: Summary of Key Findings:**

- In organoids lacking full length APC, there is more β-catenin and it localises in the cytoplasm more than in organoids with full length APC.
- Transformed APC $^{Min/+}$ organoid cysts have slightly higher levels of total β-catenin than wild type.

### 6.2.2 β-catenin and E-cadherin localise predominantly to the plasma membrane in adenomatous and serrated polyps

I have established that APC depletion causes β-catenin to move to the cytoplasm from the membrane in HCT116 cells. APC loss is an early initiating event in lesions arising via the conventional pathway of colorectal carcinogenesis. I also measured increases in the transcript and protein level of E-cadherin following PTEN loss in HCT116 cells. PTEN loss has been associated with both the conventional and serrated pathways of colorectal cancer development. I wanted to investigate whether E-cadherin levels were different in conventional versus serrated polyps.

Polyps arising via the conventional pathway of colorectal carcinoma are characterised by loss of APC and mutations in genes such as P53 and Kras. They are commonly referred to as adenomatous polyps or adenomas.

Serrated adenocarcinomas represent an alternative pathway of carcinogenesis and account for approximately 10% of colorectal cancers (Makinen, 2007). Serrated polyps differ from conventional adenomas in their typical mutation profile. The commonest defects are in the BRAF gene, present in an estimated 80% of serrated cancers (O'Brien, 2006). In addition, the CpG–Island methylator phenotype (CIMP) is also common in proximal serrated lesions (Leggett, 2010). PTEN mutation has also been associated with serrated lesions in murine models (Davies, 2014) and humans (Day, 2013).

There is a paucity of reliable predictive criteria that can identify polyps that will progress to invasive cancers. This poses challenges in determining the appropriate management and surveillance, with an estimated 35% of patients developing further adenomas within five years of removal (Winawer, 1993, Neugut, 1985). Only a small percentage of these will progress to

carcinoma. Characterising the expression patterns of proteins implicated in colorectal cancer has the potential to reveal useful tools for predicting prognosis and guiding management.

I next assessed the distribution of β-catenin and E-cadherin in a series of human colorectal polyp biopsies using immunohistochemical staining. The samples were all human colorectal polyps. The polyps were characterised by differences in histological classification. Some polyps were conventional adenomas, some were serrated adenomas and some were of mixed histology. No details of genetic or clinical characteristics were available. I aimed to determine if the cellular distribution of these proteins was comparable between conventional colorectal adenomas and serrated polyps.

A semiquantitative method was used to assess the slides. The predominant localisation of protein was classified as Membrane, Cytoplasmic or Nuclear. Nuclear β-catenin staining was scored as positive or negative. Slides were reviewed in conjunction with a consultant pathologist. Polyp scoring is displayed in Table 6.2a. Representative images of immunohistochemical slides are shown in Figure 6.3.

| Slide Number | Location of stain (E-cadherin) | Location of stain(β-catenin) | Nuclear Staining | Polyp Type |
|---|---|---|---|---|
| **1** | Membrane | Membrane | Negative | Mixed |
| **2** | Membrane | Membrane | Negative | Mixed |
| **3** | Membrane | Membrane | Negative | Mixed |
| **4** | Membrane | Membrane | Negative | Conventional adenoma |
| **5** | Membrane | Membrane | Negative | Conventional adenoma |
| **6** | Membrane | Membrane | Negative | Conventional adenoma |
| **7** | Membrane | Membrane | Negative | Conventional adenoma |
| **8** | Membrane | Membrane | Negative | Conventional adenoma |
| **9** | Membrane | Membrane | Negative | Conventional adenoma |
| **10** | Membrane | Membrane | Negative | Conventional adenoma |
| **11** | Membrane | Membrane | Negative | Conventional adenoma |
| **12** | Membrane | Membrane | Negative | Conventional adenoma |
| **13** | Membrane | Membrane | Negative | Conventional adenoma |
| **14** | Membrane | Membrane | Negative | Conventional adenoma |
| **15** | Membrane | Membrane | Negative | Serrated adenoma |
| **16** | Membrane | Membrane | Negative | Serrated adenoma |
| **17** | Membrane | Membrane | Negative | Serrated adenoma |
| **18** | Membrane | Membrane | Negative | Serrated adenoma |
| **19** | Membrane | Membrane | Negative | Serrated adenoma |
| **20** | Membrane | Membrane | Negative | Serrated adenoma |
| **21** | Membrane | Membrane | Negative | Serrated adenoma |
| **22** | Membrane | Membrane | Negative | Serrated adenoma |

*Table 6.1: The predominant localisation of E-cadherin and β-catenin was the cell membrane in conventional, serrated and mixed adenomas. Nuclear β-catenin staining was negative in all samples studied.*

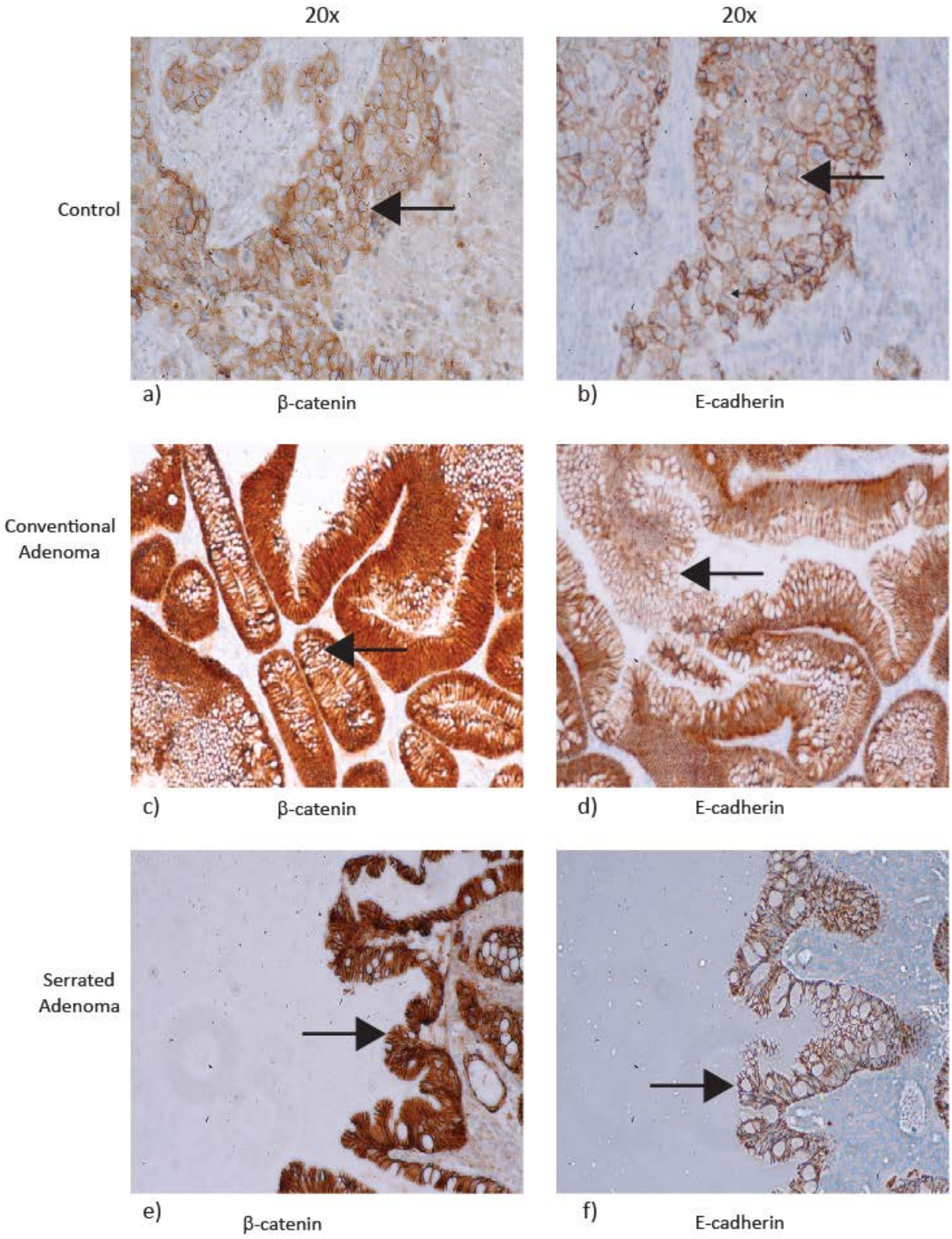



Figure 6.3: *β-Catenin and E-cadherin are localised at the plasma membrane in adenomatous and serrated colorectal polyps. Polyp biopsies were probed with antibodies targeting β-catenin or E-cadherin using immunohistochemistry. Example (20×) immunohistochemical images are shown with antibodies against β-catenin (a, c & e) or E-cadherin (b, d & f). Panels show control (a & b), a conventional adenoma (c & d) and a serrated polyp (e & f).*

In all examined slides, the predominant location of E-cadherin was the cell membrane. This was unaltered between adenomas of different histological classifications. The level of E-cadherin appeared to be similar in conventional and serrated polyps.

In all slides, the predominant location of β-catenin was the cell membrane. This was consistent in both conventional and serrated polyps. In all cases, nuclear staining for β-catenin was negative. In the polyp samples the intensity of β-catenin appeared increased. This was secondary to the tight packing of neighbouring cells. This packing results in the staining pattern appearing more diffuse. However, on closer examination the main location of β-catenin is indeed the cell membrane. The control slides came from normal colonic mucosa.

In addition to immunohistochemical staining, I also assessed the localisation of β-catenin and E-cadherin using immunofluorescent microscopy (Figure 6.4).

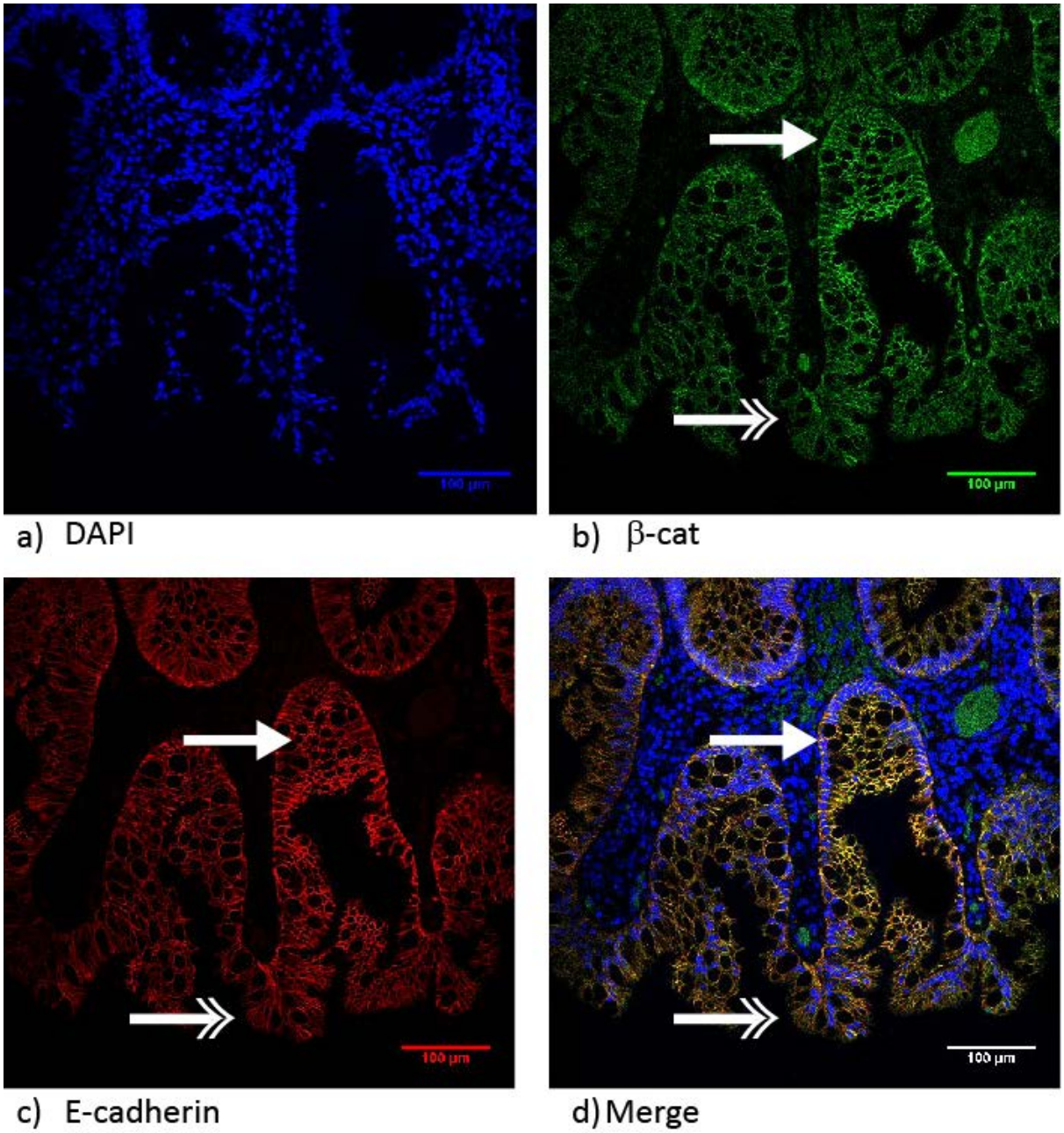



*Figure 6.4: β-Catenin and E-cadherin are located at the plasma membrane in areas of conventional and serrated adenoma in a polyp of mixed histology. A human colorectal polyp of mixed histology was probed for DAPI (a), β-catenin (b) and E-cadherin (c). The polyp has areas of both conventional adenoma (upper single headed arrow) and serrated histology (lower, double headed arrow).*

The predominant location of the β-catenin signal was at the cell membrane, although a diffuse β-catenin signal is visible throughout the cytoplasm. Nuclear localisation of β-catenin was not detected in any slides. The E-cadherin signal was primarily located at the cell membrane in all

slides. The localisation and signalling intensity of β-catenin and E-cadherin was similar in the areas of adenomatous and serrated histology.

### 6.2.2 Summary of Key Finding:

- β-Catenin and E-cadherin are predominantly localised to the plasma membrane in both conventional and serrated adenomas with no enrichment of β-catenin in the nucleus.

### 6.2.3 In polyps of mixed histology, the PTEN signal is different in areas of conventional compared with serrated histology

I next aimed to determine if the intensity or distribution of antibody detected PTEN signal was different in conventional adenomas compared with serrated polyps. Conventional adenomas are thought to arise via the conventional adenoma–carcinoma sequence. Early mutations in these lesions include loss of APC. Serrated polyps are thought to arise via an alternative pathway and differ in their mutation signature. These lesions have been associated with loss of PTEN tumour suppressor function.

A semiquantitative approach was used to assess PTEN signal: low, medium or high. The same 22 biopsy samples were used for PTEN assessments that were used for β-catenin and E-cadherin assessment (Figure 6.5). Slides were scored in conjunction with a consultant pathologist.

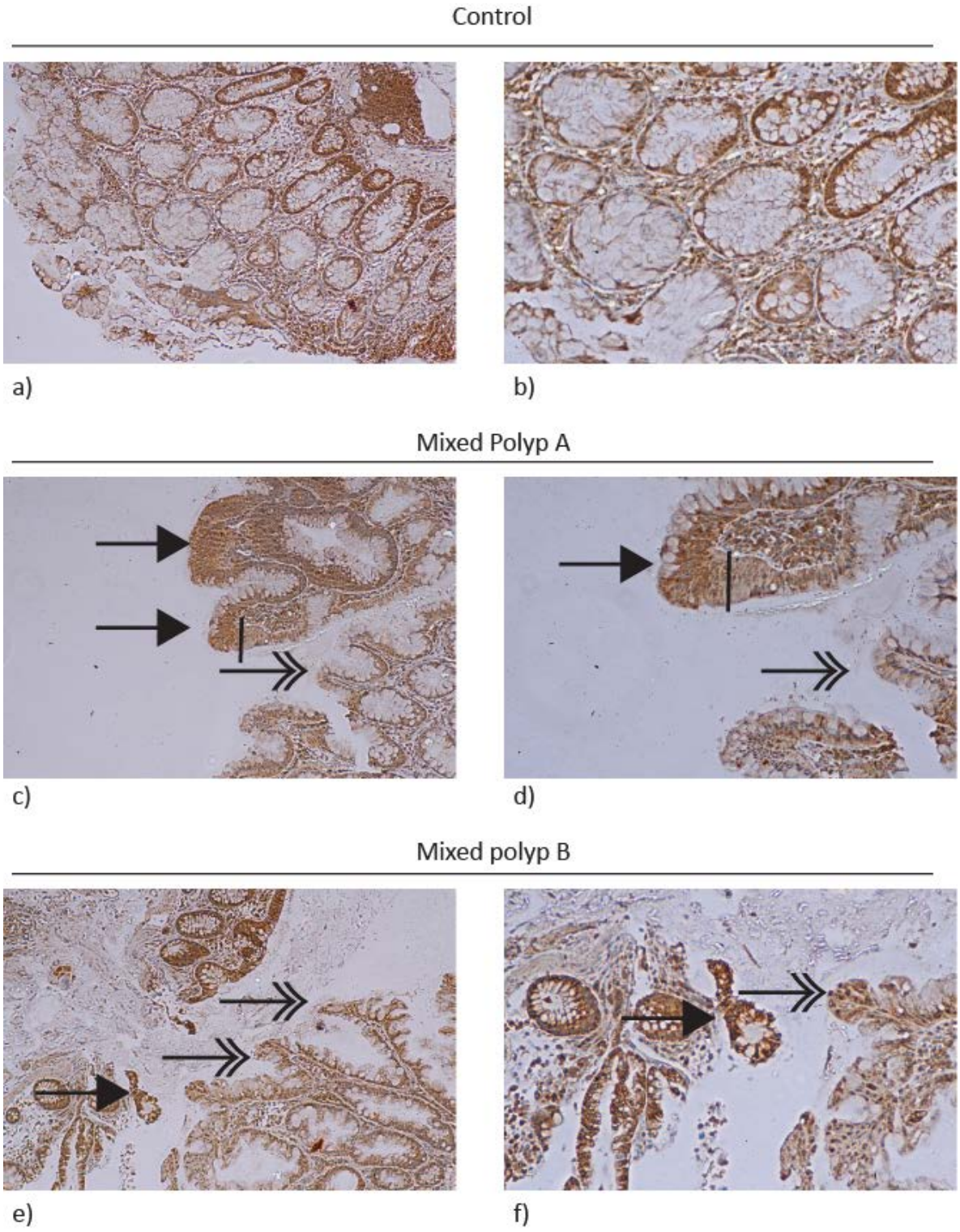



*Figure 6.5 PTEN signal intensity is higher in areas of conventional adenoma compared with areas of serrated histology. Polyp biopsies were probed with antibodies targeting* PTEN *using immunohistochemistry. Example images of polyps with mixed histology are shown (c–f) with the control in (a). Single headed arrows indicate conventional adenoma while double headed arrows show areas of serrated adenoma. PTEN signal is brown. A solid black line indicates the demarcation between normal (lighter signal) tissue on the right and conventional adenoma (higher signal) to the left of the line (c & d).*

In polyps of mixed histology, the PTEN signal was higher in the adenomatous compared with normal tissue and lower in regions of serrated histology. The PTEN signal in the majority of slides examined was of medium intensity and localised diffusely in the cytoplasm. In two of the eleven conventional adenomas, PTEN signal was higher in the adenomatous region compared with normal tissue. In four of eight serrated polyps, the PTEN signal was low in the serrated region compared with normal tissue.

The mixed polyps proved useful for comparing PTEN signal intensity in areas of conventional adenoma with regions with a serrated morphology. The PTEN signal was higher in areas of conventional compared with areas of serrated histology. Compared with normal tissue, the region of conventional adenomas had a more intense PTEN signal. This change occurred with a sharp demarcation (c & d). The demarcation point is indicated by the solid black line.

Slides were also probed with antibody against PTEN and examined using immunofluorescent microscopy. An example of a slide displaying mixed histology is shown in Figure 6.6.

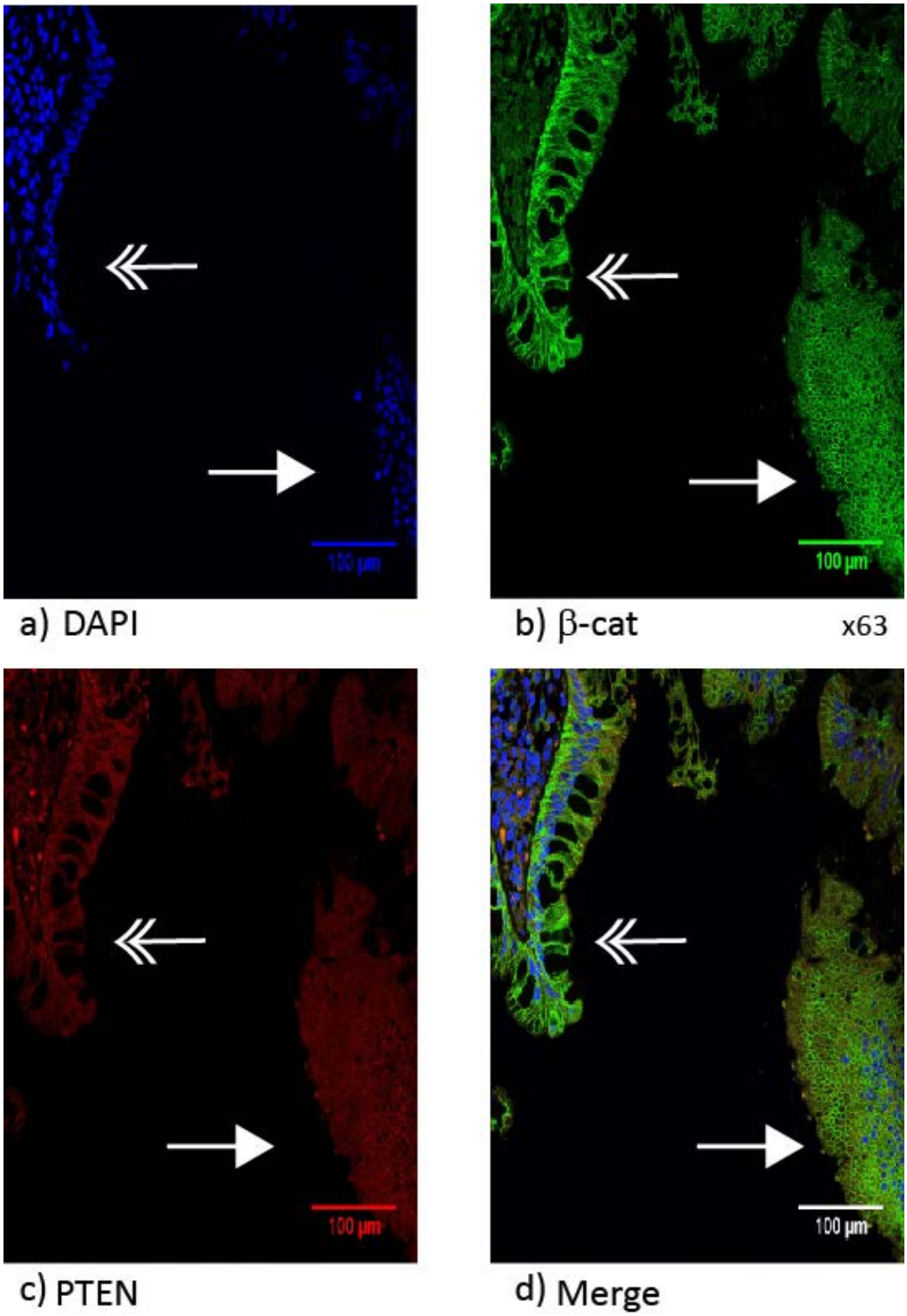



*Figure 6.6: No visible difference in the signal intensity of antibody directed against β-catenin or PTEN exists between areas of conventional and serrated adenoma in a polyp of mixed histology on immunofluorescent microscopy. A human colorectal polyp of mixed histology was stained with DAPI (a), and probed with antibodies against β-catenin (b) & PTEN (c). A merged image is in (d). The polyp has areas of both conventional adenoma (lower single headed arrow) and serrated histology (upper, double headed arrow).*

The pattern of PTEN signals between conventional and serrated polyps was consistent, with no discernible difference between polyps of different histology. The example in Figure 6.6 shows a mixed polyp with a conventional adenoma at the lower right region (single headed arrow) with an area of serrated histology in the upper left region (double headed arrow). β-Catenin is predominantly localised to the cell membrane. The PTEN stain is diffuse and similar in both the conventional and serrated regions.

### 6.2.4 Summary of Key Finding:

- PTEN is reduced in some serrated adenomas, while some conventional adenomas show a higher PTEN signal compared with normal tissue.

## 6.3 Discussion

APC loss is a very common and early event in the process of colorectal carcinogenesis. Its absence leads to deregulation of the transcriptional activity of β-catenin, often associated with nuclear enrichment of β-catenin. I detected a shift of β-catenin from the plasma membrane to the cytoplasm in colorectal cancer cell lines and aimed to establish if a similar shift was detectable in APC deplete mouse gut organoids and early stage human colorectal polyps.

Organoids derived from Apc $^{min/+}$ mice transform into cystic structures coinciding with loss of their second APC allele. I identified a higher level of cytoplasmic β-catenin in transformed compared with wild-type organoids, supporting observations in cell lines. Interestingly, the wild-type organoid had virtually no β-catenin. I would expect significant differences in the transcriptional output of β-catenin between the two and would examine transcript levels of target genes such as AXIN2 or c-myc as a next step.

The primary location of β-catenin and E-cadherin in the human polyp samples was the plasma membrane on examination of the immunohistochemistry slides. The primary location of β-

catenin in the polyps examined with an immunofluorescence microscopy was also the plasma membrane. There was also diffuse cytoplasmic presence of β-catenin, although no nuclear enrichment was evident. These were early stage polyps and perhaps a more robust translocation of β-catenin to the cytoplasm or nucleus would be detectable in more advanced lesions.

PTEN loss is implicated in both the conventional and serrated pathways of colorectal cancer. I examined the level of PTEN signal in polyps of both serrated and adenomatous histology to determine if any differences were apparent in these early stage lesions. I previously identified a higher level of E-cadherin transcript and protein expression in following PTEN depletion in HCT116 cells and aimed to assess if the E-cadherin signal differed in serrated versus adenomatous polyps.

No difference was detectable in the semiquantitative analysis of E-cadherin signal using either immunohistochemistry or immunofluorescence. PTEN signal was diffuse in both histological polyp categories. Signal intensity did appear to differ in some cases. Several serrated lesions had a light PTEN stain compared with areas of normal tissue. Mixed polyps provided an opportunity for comparison on the same slide. Serrated regions had a lower signal intensity compared with normal tissue and the adenomatous regions were characterised by a more intense PTEN signal.

PTEN loss has been associated with the serrated pathway of colon cancer. This is the reason why I wanted to compare PTEN and E-cadherin signal in conventional and serrated polyps to assess if some of my cell line results could be reproduced in human tissue. The polyp classification was based purely on histological features and no details regarding either PTEN or APC status were known. Further examination of the reduced PTEN signal in the serrated lesions could involve assessment of PTEN gene expression. Signal was not absent, simply reduced. It is unclear if this relates to defects in the PTEN protein or other factors and further experiments are required.

# 7 Discussion

In this thesis I have identified that a point mutation in residue serine 45 results in the increased interaction between β-catenin and APC. This is associated with lower levels of E-cadherin protein and transcript in the β-catenin mutant cells and a corresponding decrease in the interaction between β-catenin and E-cadherin. I have also shown that stabilised β-catenin as a result of APC loss or direct mutation of β-catenin has distinct functional consequences for the cell and this may have relevance for tumour behaviour and progression. I also measured increased levels of E-cadherin and β-catenin in cells lacking PTEN. This indicates cross talk between pathways involving two important tumour suppressor genes, APC and PTEN. I will focus further discussion of the findings of this thesis on three areas. Specifically I will discuss:

1) The effects of a single point mutation in β-catenin on its presence in complexes containing APC or E-cadherin, the functional consequences for the cell and implications for colorectal carcinogenesis.
2) The effects of PTEN loss on E-cadherin.
3) APC as a storage and transport protein for β-catenin.

## 7.1 A point mutation of residue serine 45 can change the distribution of β-catenin between complexes containing APC and those containing E-cadherin.

β-catenin is centrally implicated in colorectal cancer with vital roles in cell adhesion and transcriptional activation. Dysfunction in either role is linked with cancer. E-cadherin forms adhesion junctions with β-catenin at points of cell-cell contact and APC negatively regulates the transcriptional output of β-catenin by targeted degradation. Individually, APC or E-cadherin cannot orchestrate the regulation of β-catenin. The coordinated interplay of both is required to maintain the functional output of β-catenin within normal limits. How the relationships between β-catenin, APC and E-cadherin can be changed in colorectal cancer by different mutations is

unclear. My study provides new insights into how the distribution of β-catenin between its two main binding partners is altered by a mutation that affects its turnover and how this affects cell function.

I have shown how a mutation in a single residue (serine 45) of β-catenin causes its increased association with APC and decreased association with E-cadherin. To the best of my knowledge, this is the first time the impact of a point mutation in β-catenin on its relative interactions with both APC and E-cadherin in tandem has been measured in a colorectal cancer cell line (HCT116).

I found the mutated, Δ45 β-catenin was sequestered by APC. These cells also had very low levels of E-cadherin and correspondingly the Δ45 β-catenin associated much less with E-cadherin. In contrast, wild-type β-catenin was bound to E-cadherin most dominantly and only little was found associated with APC.

I propose that the sequestration function performed by APC acts as a sink to limit levels of free β-catenin. This function is especially important in when β-catenin is more stable and abundant. It is unclear if the sequestration of β-catenin by APC directly impacts on its association with E-cadherin or if mutant β-catenin is impaired in its ability to bind E-cadherin. The distribution of β-catenin with either E-cadherin or APC, which can either sequester (mutant) or degrade (wild-type) β-catenin appears to be able to regulate the transcriptional activity of β-catenin. Despite, differences in total levels of APC, E-cadherin and β-catenin, or its susceptibility to degradation did not correlate with significant differences in transcription of AXIN 2 between the cell lines studied.

A number of past studies have considered the effects of stabilised β-catenin on the interaction with E-cadherin in HCT116 cells. Chan and colleagues (Chan *et al.* 2002) found an increased association of wild-type β-catenin with E-cadherin compared to a low level of interaction between mutant β-catenin and E-cadherin. These findings are in accordance with mine.

Measurements of the association of β-catenin with APC was not made. In addition, they found wild-type β-catenin to be associated with the cell membrane but mutant β-catenin to have a diffuse cytosolic localisation on immuno-fluorescence microscopy. These findings are in contrast to mine. I found both mutant and wild-type β-catenin to be predominantly located at the membrane under control conditions using immuno-fluorescence microscopy and biochemical cell fractionation. I believe the discordance between my results and those of Chan could be related to cell density. Localisation of β-catenin was assessed using microscopy of very low density cells by Chan, which can influence β-catenin localisation. They detected more nuclear β-catenin in a nuclear fraction of mutant compared to wild-type cells based on absolute levels. My results measuring the relative distribution of β-catenin in the cytosol, membrane, nucleus and cytoskeleton showed no difference in the relative amount of nuclear β-catenin between all cell lines. It is likely that the mutant cell line has a greater absolute amount of nuclear β-catenin because the overall levels are higher than in the wild-type cells. Nonetheless, this did not cause a detectable difference in transcription of AXIN 2.

Atomic force microscopy measurements showed that E-cadherin bond strength is lower in HCT116 expressing mutant β-catenin compared to those expressing wild-type β-catenin and this correlated with lower levels of E-cadherin (Bajpai *et al.* 2013) although they determined that mutant β-catenin was capable of binding E-cadherin. This is in agreement with my findings but the authors did not measure the interaction of β-catenin with APC. An increased association between β-catenin and APC has been identified in MDCK cells using exogenously expressed β-catenin with N-terminal deletions that include Ser45 (Barth *et al.* 1997). In this study E-cadherin levels were found to change little in response to expression of the mutant β-catenin proteins. In a similar study using AtT20 cells, N-terminally deleted β-catenin was also found to be bound more to APC than wild-type β-catenin. (Munemitsu *et al.* 1994).

A recent study by Huels and colleagues made interesting discoveries relating to the interactions between β-catenin and E-cadherin in the mouse intestine. Mice with exon 3 deletion of β-catenin were generated. This region contains the phosphorylation sites required for APC targeted destruction so the β-catenin is not degradable. These β-catenin mutant mice took longer to develop small intestinal lesions than those with β-catenin mutations in either β-catenin alleles or APC loss. A single activating β-catenin mutation was not enough to trigger transformation of the colon, which had higher levels of E-cadherin than the small intestine. A greater level of interaction between β-catenin and E-cadherin was found in the colon and this was true for the mutated (stabilised) and wild-type β-catenin. The authors proposed that E-cadherin can act as a sink for mutant β-catenin and this limits the potential for β-catenin to induce transformation (Huels *et al.* 2015). However, there was no assessment of the interaction of β-catenin with APC. These findings support my hypothesis that APC and E-cadherin can bind and sequester β-catenin to maintain its transcriptional output within set limits. Importantly, I found that APC is a more effective sink for β-catenin than E-cadherin.

A common result from my studies and those by Barth and Munemitsu is that N-terminal deletion or point mutation of β-catenin that cause its stabilisation leads to its increased association with APC. More specifically, I have shown that a single point mutation of serine 45 is enough to cause this effect. The N-terminus contains a number of key phosphorylation sites essential for the degradation of β-catenin. Serine 45 is phosphorylated as a priming step for sequential phosphorylation at residues 44, 37 and 33 prior to ubiquitination and proteosomal degradation (Yost *et al.* 1996, Hart *et al.* 1999, Liu *et al.* 2002). Absence of serine 45 does not appear to preclude binding of β-catenin to APC but it does it non-degradable. The mechanism responsible for sequestration cannot be determined from my experiments.

One possibility is that once bound to APC the mutant β-catenin becomes physically “stuck” and cannot be released creating sequestration effect by default. An alternative is that APC assumes

an active role in regulating β-catenin when degradation is not possible. Although these two scenarios both result in the sequestration of β-catenin by APC they are different. The first implies that phosphorylation of serine 45 is an essential prerequisite to trigger the release of β-catenin from APC so once bound they cannot be separated. The second implies that APC can take on a formal role in the sequestration of β-catenin to maintain levels of free β-catenin within acceptable limits. Here, the release of β-catenin from APC is physically possible but in order to act as a sink APC is actively holding on to the β-catenin. Rather than enforced sequestration this represents a deliberate, functional sequestration resulting in the ability to control free β-catenin levels within acceptable limits.

I would investigate this further by extending my β-catenin overexpression experiments. I would express mutant tagged β-catenin in the mutant cell line, which already has a large pool of β-catenin sequestered by APC. I would then perform an APC IP at staggered time points. I would aim to measure if the exogenous tagged β-catenin becomes increasingly associated with APC over time, at the expense of the endogenous mutant β-catenin. If so, this would signify that mutant β-catenin could indeed cycle off APC and the relationship was therefore deliberate as opposed to enforced by the β-catenin becoming “stuck”. As a further extension of the over expression experiments I would over express E-cadherin in the cell lines studied to assess if a greater pool of β-catenin associated with it. This is especially relevant in the mutant cell line, which had very low levels of E-cadherin.

The mutant cells in my study had higher expression levels of both β-catenin and APC. It is unclear from my experiments if higher β-catenin can lead to more APC. Logically this would seem appropriate as higher levels of β-catenin may require more APC to regulate it. I also found that upon depletion of β-catenin, APC levels fell. The two appear to be linked however further experiments would be required to clarify this relationship.

I also detected lower levels of mutant β-catenin in association with E-cadherin than wild-type in agreement with previous findings (Chan *et al.* 2002). I investigated this further and found that this correlated with low transcript levels of E-cadherin. I identified a further effect of mutant β-catenin on E-cadherin transcription. Expressing exogenous mutant β-catenin led to a reduction in E-cadherin transcripts regardless of whether the endogenous β-catenin was mutant or wild-type. Expressing exogenous wild-type β-catenin on the other hand, caused an increase in E-cadherin transcripts. Although these finding reveal complex relationships between β-catenin and E-cadherin at the transcript and protein level, they do not explain why mutant β-catenin does not appear to bind E-cadherin as well as wild-type. Following induction of mutant β-catenin in the mouse colon, mutant β-catenin was increasingly bound to E-cadherin over wild-type β-catenin over time when assessed by co-immunoprecipitation at staggered time points. This implies that binding *in vivo,* can occur. Unfortunately, concurrent measurements of β-catenin interaction with APC were not made making it difficult to compare these results to mine (Huels *et al.* 2015). I found more mutant β-catenin in association with APC but not E-cadherin. In both, my study and that of Huels, either APC or E-cadherin preferentially sequester the mutant non-degradable β-catenin. It is unclear if this is due to some structural consequence of the mutation that results in increased binding of mutant β-catenin to either APC or E-cadherin.

Findings from my study and that of Chen found mutant β-catenin less able to bind to E-cadherin and HCT116 cells. Correspondingly, cells expressing only mutant β-catenin exhibit weaker E-cadherin bond strength than those expressing only wild-type β-catenin (Bajpai *et al.* 2013).. This could potentially be related to structural changes induced by deletion of serine 45 that in turn could reduce the ability to bind E-cadherin. Mutations affecting β-catenin in tumours tend to impact on specific residues in the N-terminal region or involve deletion of exon 3, which contains these residues (Ilyas *et al.* 1997, Morin *et al.* 1997, Johnson *et al.* 2005, Segditsas and Tomlinson 2006). Importantly this region of β-catenin is distinct form the armadillo region that binds to E-cadherin and is not expected to directly impact on E-cadherin binding. Phosphorylation sites in

β-catenin that are involved in regulating its binding to E-cadherin are at the opposite, C-terminal end of the protein. Specifically, phosphorylation of Serines 686 and 692 increases the stability of the interaction between β-catenin and E-cadherin (Huber and Weis 2001), while phosphorylation of Tyrosine 654 decreases the affinity of β-catenin for E-cadherin, which may enhance β-catenin transcriptional activity. (Piedra *et al.* 2001). The C-terminal domain of β-catenin is known to affect its ability to participate in either transcriptional regulation or adhesion. Back folding of the C-terminus blocks the armadillo region compromising binding to E-cadherin. In this state, β-catenin is transcriptionally active whereas when bound to alpha-catenin its C-terminal region is not folded back permitting binding to E-cadherin (Gottardi and Gumbiner 2004). It is conceivable that deleting Serine 45 causes a conformational change in β-catenin that also impedes E-cadherin binding. However, it is not at all clear how interfering with the N-terminal domain can block the armadillo region and reduce binding to E-cadherin to produce an effect similar to the back folding of the C-terminus. Similarly, whether deleting entire stretches from the N-terminal domain of β-catenin causes the changes in the stability of interactions with E-cadherin in a similar manner to point deletion of Serine 45 will require detailed structural analysis.

Another regulatory step that affects the interaction between β-catenin and E-cadherin is the phosphorylation of E-cadherin, which can increase as well as decrease its affinity for β-catenin (Gottardi and Gumbiner 2004) (Piedra *et al.* 2001). An important kinase that facilitates these events it GSK3β. The strength of E-cadherin bonds in HCT116 cells expressing wild-type β-catenin decreased to a level comparable to HCT116 cells expressing mutant β-catenin upon depletion of GSK3β whereas there was no change when GSK3 was depleted from cells expressing only mutant β-catenin (Bajpai *et al.* 2013). This observation and my own preliminary observations lead me to speculate about why mutant β-catenin associates less well with E-cadherin than wild-type. Preliminary experiments showed that when mutant β-catenin is sequestered by APC, Axin could be co-immunoprecipitated by APC (data not shown) suggesting

that other components of the destruction complex are associated with the sequestered β-catenin. If GSK3β is one of these, its availability to phosphorylate E-cadherin may be reduced so that E-cadherin has reduced affinity for β-catenin. This could explain why mutant β-catenin binds poorly to E-cadherin. At present this is merely speculation. To test this theory I would compare the presence of GSK3β in an APC and β-catenin IP in mutant versus wild-type cells.

Stabilised β-catenin can result from either N-terminal β-catenin deletions or of a point mutation in serine 45 while APC loss also results in stabilised β-catenin. Mutations of APC or direct mutation of β-catenin can both lead to stabilised β-catenin but the functional consequences of either mutation are different. I have shown that mutant, stabilised β-catenin can be sequestered by APC and has little interaction with E-cadherin. That can explain why AXIN 2 transcription is equivalent in cells expressing only wild-type or mutant β-catenin. In both of these cell types, the predominant localisation of β-catenin is the cell membrane. This is consistent with the observation that APC depletion causes a redistribution of β-catenin to the cytoplasm and a concomitant significant increase in AXIN 2 transcription, regardless of whether β-catenin is mutant or wild-type and without or only modest increases in total β-catenin levels. I also showed that depleting E-cadherin caused a similar redistribution of β-catenin but was accompanied by a less dramatic effect on transcription of Axin. These findings indicate that the localisation of and/or the availability of free β-catenin is more important than total abundance for stimulating transcriptional activation. This is consistent with the idea that elevated levels of E-cadherin found in the mouse colon (compared to small intestine) can limit transformation caused by an activating β-catenin mutation (Huels *et al.* 2015). Huels' study suggests that E-cadherin provides a buffering effect of the β-catenin mutation. My findings show APC can also perform this buffering function. The binding of β-catenin to either APC or E-cadherin limits the levels of free, transcriptionally active β-catenin, which I have shown accumulates in the cytoplasm. This supports the idea that regulation of β-catenin by both APC and E-cadherin is critical and impacts on cancer progression.

Although a Serine 45 point mutation of β-catenin and APC loss both lead to stabilisation of β-catenin, the consequences on transcriptional output of β-catenin are different due to the ability of APC to sequester β-catenin. The cells expressing mutant β-catenin proliferated faster but had an equivalent level of AXIN 2 transcription compared to cells expressing wild-type β-catenin. To investigate this more closely I would measure the transcription levels of other β-catenin/Wnt target genes and also dissect in more detail the relative abundance of binding partners in complexes formed by mutant versus wild-type β-catenin (for instance using Mass Spectrometry).

Loss of APC has additional functional consequences. APC depletion in all cells examined leads to a reduction in cell migration and this was mainly due to a loss of direction. The overall negative effect ton migration may increase the residency time of an APC mutant cell within a crypt, increasing its likelihood of further accumulation of mutations. . Thus it is not only loss of the ability of APC to regulate β-catenin that has relevance in cancer but also functions related to interactions facilitated by its C-terminus, for instance its effect on microtubules.

These findings provide insight into why APC loss in colorectal tumours is very common while β-catenin mutations are comparatively rare. APC loss is a very common finding in both colorectal tumours and early stage lesions indicating that it is an early event. Some tumours lacking APC mutation have been found to have activating mutations of β-catenin (Miyaki *et al.* 1999, Johnson *et al.* 2005) and it has been suggested that APC and β-catenin mutations in tumours are mutually exclusive (Morin *et al.* 1997) (Sparks *et al.* 1998). Recent data challenge this assertion by identifying mutations in multiple Wnt pathway genes in tumours that harbour APC mutation (Cancer Genome Atlas 2012). This suggests multiple abnormalities affecting the Wnt pathway may act synergistically to provide the selective advantage necessary to facilitate tumour progression.

β-catenin mutations are far less common than loss of APC and are characterised by point mutation of N-terminal residues or deletion of exon 3 (Ilyas *et al.* 1997, Morin *et al.* 1997, Johnson *et al.* 2005, Segditsas and Tomlinson 2006). They are particularly associated with Hereditary non-polyposis coli (HNPCC) (Miyaki *et al.* 1999) but far less common in sporadic cancers (Johnson *et al.* 2005). β-catenin mutation in HNPCC may result from the mutator phenotype that characterises these lesions due to defects in mismatch repair genes. In addition some authors have identified a higher number of small sporadic adenomas to harbour β-catenin mutations than sporadic cancers (Samowitz *et al.* 1999). This suggests that lesions with mutations in β-catenin may be less likely to progress than those with loss of APC.

This theory fits with some of my findings. Lesions with stabilising mutations of β-catenin may be less likely to progress if APC remains intact and sequesters the mutant β-catenin thus limiting transcriptional activation. Loss of APC or mutations that produce APC protein unable to bind and sequester β-catenin, on the other hand result in increased β-catenin transcriptional activation together with migration defects. Both could increase the risk of cancer progression. It is unclear if mutant β-catenin has altered interaction with APC *in vivo.* This could be investigated further by producing organoids from mice with an activating β-catenin mutation and measuring the interaction between mutant β-catenin and APC using Co-IP to allow comparing their relative association with that in wild-type organoids. Mutation of β-catenin is more common in other tumour types such as hepatocellular carcinoma or pseudo papillary pancreatic tumours than in colorectal cancer. In these tumours reduced E-cadherin levels are associated with increased transcription of β-catenin target genes. Mutation of β-catenin and loss of E-cadherin may act synergistically here to promote tumour progression. (Huels *et al.* 2015). In colorectal cancer down regulation of E-cadherin has been associated with late stage lesions and the EMT process.

The finding that β-catenin transcriptional output in HCT116 cells with activating β-catenin mutation only increases following APC depletion is consistent with the “just right” hypothesis.

This theory relates to different levels of Wnt signalling produced by different APC mutations and proposes that there is an optimal amount of Wnt signalling for cells to have the growth advantage required to form a tumour (Fodde and Brabletz 2007, Buchert *et al.* 2010). My findings indicate that a stabilising β-catenin mutation alone may not be sufficient to stimulate β-catenin transcriptional output but that additional changes such as loss of APC are required to produce significant increases in transcription of target genes, such as AXIN 2. This increased transcriptional output combined with the consequences of APC loss on cell migration, may hasten the progression of tumours. This may explain why β-catenin mutations in colorectal tumours are less common than those harbouring APC mutations.

With further refinement, these findings have important implications for the management of patients with cancer. For example if a patient has a polyp removed a conundrum exists as to whether they enter a surveillance programme or require formal surgical resection to enable full pathological staging by removing both the lesion site and lymph nodes. Such a radical procedure may however be unnecessary and poses significant risk to the patient. Analysis of the lesion for the presence of mutations of APC or β-catenin combined with analysis of β-catenin localisation or transcriptional output could be used to guide therapy or surveillance intensity. As shown, β-catenin mutations may be less likely to progress in the absence of additional mutations whereas an APC mutation may immediately cause more functional aberrations making progression more likely. Of course I am extrapolating my findings here but with continued technological progression and understanding of molecular defects in cancer such targeted treatments may one day be possible to match the patient and their tumour.

**7.2 The effects of PTEN on E-cadherin**

I have shown that PTEN can cause changes in the levels of both β-catenin and E-cadherin. I believe two separate mechanisms are responsible for this. The lipid phosphatase activity of PTEN is likely to be responsible for changing levels of β-catenin while the physical presence of the PTEN protein appears to impact on the level of E-cadherin.

I have shown that PTEN (-/-) cells express higher amounts of E-cadherin protein and transcripts than PTEN (+/+) cells. I have also shown that depletion of PTEN from PTEN (+/+) cells causes increases in the transcription and protein level of E-cadherin. These data were supported by the observation that expression of exogenous PTEN protein in PTEN (-/-) cells leads to reactivation of E-cadherin transcription.

These findings are in contrast to a number of previous studies that associate down regulation of PTEN with decreased E-cadherin expression in a number of tumour types including ovarian, melanoma and nasopharyngeal (Hao *et al.* 2012, Zhang *et al.* 2013, Huang *et al.* 2014).

There is evidence that PTEN associates with adherens junctions. Studies using MDCK cells found that PTEN stabilised E-cadherin at adherens junctions but it did not alter overall levels of β-catenin or E-cadherin (Kotelevets *et al.* 2001). A further study by the same group showed that PTEN could be co-immunoprecipitated with E-cadherin and β-catenin. Here, PTEN had an indirect link to β-catenin via the scaffold protein MAG1-1b (Kotelevets *et al.* 2005). Similarly, both PTEN and PI3K were identified in association with E-cadherin and β-catenin at adherens junctions in a pancreatic cancer cell line. In this case the phosphatase activity of PTEN was shown to play a role in the phosphorylation of β-catenin (Vogelmann *et al.* 2005).

Although the exact role of PTEN in the regulation of adhesion junction components E-cadherin and β-catenin is unclear, there is evidence suggesting that these proteins co-localise in some cells. I found transcriptional repression of E-cadherin by PTEN in HCT116 cells. Interestingly the

transcriptional repression of E-cadherin was produced by transfection of a panel of PTEN proteins, including those no phosphatase function. This suggests that the physical presence of the PTEN protein is important rather than a specific enzymatic function. To establish a mechanism that explains these observations will require further experiments. A first step would be to identify the presence of PTEN in a complex with E-cadherin and β-catenin using co-immunoprecipitation. It is possible that the interaction of PTEN and β-catenin via MAGI-1b may be dependent on a PDZ domain. In that case, expression of a PTEN protein lacking a PDZ domain may be expected to affect its relationship with E-cadherin or β-catenin. Measuring E-cadherin and β-catenin levels following introduction of such a PTEN mutant protein could confirm this idea. I hypothesised that the relationship between PTEN and E-cadherin may involve Snail but I could not detect any change in the amount of Snail in response to PTEN expression. To take this further I would also assess other relatives of Snail involved in the EMT process such as Slug and Twist.

The mechanism mediating the influence of PTEN on E-cadherin transcription may also involve a feedback loop regulating adherens junctions. A close association with the adhesion junction complex may place PTEN in a position to indirectly regulate transcription of some of the junctional components. Close association of PTEN with the junctional complex may act as a platform to recruit other proteins to the adherens junction that are released when the junction disassembles or is not fully functional. This in turn may act as a signal for the requirement for more E-cadherin. Release of PTEN and such additional proteins may stimulate E-cadherin transcription to maintain junctions. In the PTEN (-/-) cells such 'transcription activating agents' are unable to associate with PTEN or the adherens junction and therefore can stimulate transcription of E-cadherin. This speculative hypothesis will have to be tested by further experiments.

I have shown that PTEN affects the levels of both β-catenin and E-cadherin identifying potential cross talk between two common tumour suppressor genes, PTEN and APC.

This is potentially important in cancer and several studies have shown combinations of mutations in these two tumour suppressor genes can enhance cancer progression. Loss of APC alone caused uterine hyperplasia but no malignant transformation in a mouse model of endometrial cancer. PTEN loss could initiate invasive lesions and loss of both tumour suppressor genes together hastened progression and caused more aggressive lesions than loss or mutation of either alone (van der Zee *et al.* 2013). In murine models of colorectal cancer, PTEN loss alone caused no alteration of epithelial architecture but in combination with APC loss mice developed adenocarcinoma (Marsh *et al.* 2008). This indicates that effects of PTEN loss may require additional dysfunction in β-catenin transcriptional activity to cause tumour genesis. A separate study by the same group found that in mice predisposed to adenoma development ($APC^{fl/+}$) with PTEN and KRAS mutations developed adenocarcinoma with no metastasis. In contrast, APC wild-type mice carrying mutation in PTEN and KRAS developed invasive metastatic cancers with a serrated histology. These findings indicate that the relationships between combinations of mutations and there functional consequences are complex. In addition, the effects of specific mutational signatures may act in a tissue dependent manner given the findings in endometrial versus colon cancer in mouse models described above.

These studies indicate that crosstalk exists between the APC and PTEN pathways in tumours. My data support this at a molecular level. I have shown that changes in the amounts of E-cadherin and β-catenin occur in response to PTEN depletion. These two proteins are also implicated in colorectal cancer following APC loss. Further characterisation of the of the cross talk at the molecular level will help elucidate how mutations in PTEN and APC can synergise to influence tumour characteristics.

PTEN mutations in human cancers have been associated with serrated lesions (Day *et al.* 2013). Consistently, I found lower PTEN signal in areas of serrated histology in some early stage human colorectal polyps. Analysis of a larger number of polyps and a broader range of tumour stages is required to characterise this further. In addition, the corresponding levels and localisation of E-cadherin and β-catenin and mutational status of APC will need to be measured to determine how the findings in cell lines relate to situations in tumours.

My data showed that PTEN can affect levels of both β-catenin and E-cadherin in HCT116 cells. The effects on E-cadherin appear to be dependent on the physical presence of PTEN protein to repress E-cadherin transcription. The link between PTEN and adherens junctions is important in cancers as adherens junctions are often down regulated during the process of EMT and cancer progression. I have shown an increase in E-cadherin when PTEN is depleted. Clarification of the mechanism responsible may allow development of strategies to manipulate E-cadherin levels in tumours, dampening the process of EMT and halting tumour progression.

In the cell lines studied I measured effects of loss or re-expression of PTEN. This all or nothing approach may not reflect the situation in tumours, where mutations in the protein or post translational modifications may influence the protein activity as opposed to a complete absence of the protein. Modifications to the protein could potentially influence tumour progression or influence association with a particular histological category. Further work is required to assess if specific mutations can impact on β-catenin and E-cadherin in a similar manner to complete loss of the PTEN protein

### 7.3 A role of APC in 'storing' and transporting β-catenin

I have shown that APC can sequester stabilised β-catenin. This can regulate transcriptional output of β-catenin. Similar attributes have been assigned to E-cadherin in mouse colon and this has been shown to limit tumour genesis (Huels *et al.* 2015). The coordinated actions of APC and E-cadherin thus can regulate β-catenin and help to maintain intestinal homeostasis. Dysfunction of any component of this triad can predispose to cancer.

APC has many functions and targeting for degradation is usually considered its primary function in the regulation of β-catenin. I propose that APC potentially has as a primary role in the regulation of β-catenin that involves storage and transport and that under certain conditions targeting for degradation is secondary. This is a speculative hypothesis and further experiments would be required to clarify this. But I will outline some justification for this proposal.

Stabilised β-catenin is sequestered by APC. If this sequestration is active and serves as functional sequestration to regulate β-catenin as opposed to enforced due to the mutant β-catenin being physically "stuck", then this would provide a second method whereby APC can control free levels of cellular β-catenin. When precipitating APC, more mutant β-catenin was bound to APC than wild-type. Nonetheless, there was a significant amount of wild-type β-catenin found in association with APC. If the relationship between APC and β-catenin serves only to degrade β-catenin, one would assume it was transient so that relatively little β-catenin is bound to APC at any one time. In addition, the regulation of β-catenin levels is maintained within tight limits to ensure normal cell function and deviations from this caused by stabilising mutations or APC loss can predispose to cancer. Indeed, transcript levels of β-catenin in the mutant and wild-type cell lines were very similar. If a large amount of β-catenin was bound to APC for degradation, this could suggest that the cell has somehow "over shot" its acceptable concentration of β-catenin, necessitating large scale degradation. Given the potential consequences of over stimulation of β-catenin transcriptional activity this seems like a risky strategy for the cell.

If the primary function of APC is related to storage, it would provide the cell with a pool β-catenin that can be released should it be required by the cell. A Wnt signal can then provide an additional mechanism to top up this β-catenin pool should the cell require it, by inhibiting the destruction complex.

I found that the interaction between β-catenin and APC takes place at the membrane of the cell. This is in keeping with previous findings that the destruction complex is associated with the membrane following inhibition by Wnt signalling (Li *et al.* 2012). If indeed APC performs a role in the transport and storage of β-catenin, a plasma membrane associated location would seem logical as it is in close proximity to E-cadherin. This would allow exchange of sequestered β-catenin from APC to E-cadherin and vice versa. A study by Klingelhofer and colleagues support this idea. They showed that β-catenin can cycle on and off E-cadherin at the membrane but this process is impaired in APC deficient cells (Klingelhofer *et al.* 2003). Further support for a role of APC in the regulation of adherens junctions was identified following expression of full length APC in SW480 cells. This stimulated a translocation of β-catenin from the nucleus to the plasma membrane. In addition, E-cadherin also moved to the membrane. This is consistent with a role for APC in the transport of components of the adhesion complex (Faux *et al.* 2004).

I also found that β-catenin redistributed to the cytoplasm following APC depletion but was not enriched in the nuclear. However, AXIN 2 transcription increased under these conditions. Previous authors have identified a role for APC in the nuclear export of β-catenin (Rosin-Arbesfeld *et al.* 2000, Rosin-Arbesfeld *et al.* 2003). APC lacking a nuclear export signal remained capable of regulating β-catenin transcriptional activity indicating a role for APC ion the sequestration of nuclear β-catenin (Neufeld *et al.* 2000). This supports a role for APC in both the active transport and storage of β-catenin as a means of regulating transcriptional output.

This theory is speculative but my results and those of others indicate that APC could potentially regulate β-catenin by its deliberate sequestration. It could also fulfil a role in the transport of β-

catenin between cell regions to bring β-catenin in close proximity to E-cadherin while limiting the free pool available to activate transcription. Additional layers of regulation are added by the targeted degradation of β-catenin if levels are too high and by inhibition of the destruction complex in response to a Wnt ligand. The presence of an APC-associated, stored pool of β-catenin may allow finer tuning of β-catenin levels than a simple model with and on and off state for the degradation complex. Fine tuning would fit with the theory that the correct level of β-catenin transcriptional output is required for carcinogenesis, in keeping with the “just right” model. Such tight regulation is likely also required for normal homeostasis. A role for APC as both a storage depot and transporter that can shuttle β-catenin between different compartments could be an effective mechanism to achieve this fine tuning.

# 8 Future Experiments

This project has raised a number of questions that I would like to investigate in more detail. I would like to determine if mutant β-catenin was capable of cycling on and off of APC or if it is simply physically "stuck". This could potentially distinguish between a passive role of APC in sequestering β-catenin statically (indicated by slow cycling) or a more active role in dynamically sequestering β-catenin so that can be regulated independently (indicated by more rapid cycling). To do this I would exogenously express tagged mutant β-catenin in cells expressing either mutant or wild-type β-catenin that can be induced rapidly. Performing APC immunoprecipitations at different times after inducing expression of the exogenous β-catenin would reveal how quickly tagged β-catenin replaced the endogenous wild-type or mutant β-catenin bound to APC over time. Similarly, investigating the β-catenin that can be co-immunprecipiated with E-cadherin would reveal the relative interactions of wild-type β-catenin and mutant β-catenin with E-cadherin.

I would repeat the molecular analysis of the relationships between APC, β-catenin and E-cadherin in organoids. They represent a more physiologically relevant system and initially I would compare levels and interactions between β-catenin, E-cadherin and APC from wild-type and APC $^{Min/+}$ mice. I would also like to carry this out on organoids from mice carrying a stabilising β-catenin mutation. Ultimately I would like to investigate these relationships in organoids derived from human tissue. Tumour samples with either APC or β-catenin mutations would be compared to clarify differences at the molecular level. This system would also provide a platform for drug screening studies.

I would also investigate the relationship between E-cadherin and PTEN in more detail. I would start by attempting to Co-IP PTEN with β-catenin and E-cadherin. If this was successful I would express a mutant PTEN protein lacking a PDZ domain to measure if this region was necessary for the interaction between PTEN, β-catenin and E-cadherin. If I was able to prove interaction of β-

catenin, E-cadherin and PTEN in HCT116 cells I would analyse a Co-IP study using mass spectrometry to highlight any other proteins present in the complex. These proteins would then be candidates for involvement in the transcriptional repression of E-cadherin and would be studied further.

A larger scale mass spectrometry based screen of the wild-type and mutant cells would also be undertaken. Multiple differences exist between these cells in morphology, function and biochemical properties. The only genetic difference between the cells is that one expresses mutant and the other wild-type β-catenin. One may assume that much of the functional and biochemical differences between the cells result from differential output of β-catenin transcriptional activity. A screen to determine differences in protein expression between the cells could then be used to focus a more specific analysis. For example if proteins known to be involved in cell adhesion were enriched more in the wild-type than the mutant cells then these could be considered further with the aim of explaining differential interaction between β-catenin and E-cadherin. A similar approach could be taken to other cell functions. This would provide more insight into how molecular differences influence their relevant signalling pathways and in turn, cell function.

I would extend my experiments to include a broader assessment of Wnt target genes and assess differences between the mutant and wild type cell lines. I examined Axin 2 which is a recognised Wnt target gene and is frequently used to assess β-catenin transcriptional activity. I would measure transcription of a panel of other β-catenin target genes using qPCR. Other reporter systems exist such as TOP flash. This represents a more basic determination of an either on or off state. I feel that qPCR measurements of target genes is more sensitive given the complexities of β-catenin transcriptional activity.

I would also carry out sequencing of the mutant and wild type cells I have used. Although these cells are isogenic and differ only in their expression of β-catenin it must be borne in mind that

they are cancer cells of the mutator phenotype. Sequencing would clarify if the existence of further mutations are contributing to experimental findings.

Lastly I would extend my analysis of human polyps to include a variety of stages as opposed to only early stage lesions that I studied. Detailed information about the mutations in the samples in APC, PTEN or KRAS would also allow comparison of the findings to those identified in the cell line studies. This may highlight opportunities for new diagnostic or therapeutic approaches.

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# Appendix 1

## Analysis of β-catenin distribution using immunofluorescence microscopy

### Introduction

The initial approach in my project involved using immunofluorescence microscopy to measure the distribution of β-catenin within the cell. By determining location I hoped to be able to infer which of its most common binding partners it was likely to be associated with and the function that it was playing. For example β-catenin at the membrane may signify a role in cell adhesion while nuclear β-catenin may imply a signalling function. I aimed to assess if the distribution of β-catenin was responsive to biochemical manipulation of the levels of its binding partners. I planned to measure the distribution of β-catenin in both HCT116 colorectal cancer cells and in mouse gut tissue.

### Cell compartment identification and image analysis

To address measurement of intracellular distribution of β-catenin using immunofluorescent imaging, I had to establish a method of visually identifying the cell membrane and nucleus using markers so that I could extract the relevant data from the images. Numerous methods were attempted and failed. I found DAPI to be a suitable stain to identify the cell nucleus. I tried several aproaches to identifying the cell membrane. The best visual representation of the cell membrane was achieved using antibody directed against β-catenin. Considering that β-catenin was to be the focus of my measurements, I deemed it inapproprate to use this to identify the cell membrane compartment. I compared phalloidin stain with an antibody directed against $Na^+/K^+$ ATPase. Both of these prodced a good representation of the cell membrane. Phalloidin was a better choice as it is a stain as opposed to an antibody. This resulted in more flexibility

when using other antibodies as more options would be available for the specific species of secondary antibodies.

Alternative membrane stains such as wheat germ agglutinin were unsuccesful as it stained the nuclear membrane as opposed to the plasma membrane. I also attempted to use cytoplasmic stains to identify the cytoplasm. These were unsuccesful as they produced a diffuse non-specific staining pattern. I therefore decided to use DAPI to identify nuclei and phalloidin to mark the cell membrane.

Using these markers to identify visually the relevant cell compartments, a threshold can be applied to their signal intensity using image analysis software. This allows the creation of a “mask”, representing an area overlaying the region of interest. In this way, masks representing the membrane and nuclear compartments can be created. Combining these two masks and inverting creates a mask for the remaining cytoplasmic compartment. Examples of these “masks” can be seen below in Figure A1 and show good correlation with regions identified visually with the phalloidin and DAPI stains, respectively. Using image analysis software the masks can be overlain on the original image. The signal intensity from the protein of interest within the area covered by the overlying “masked” region can then be calculated.

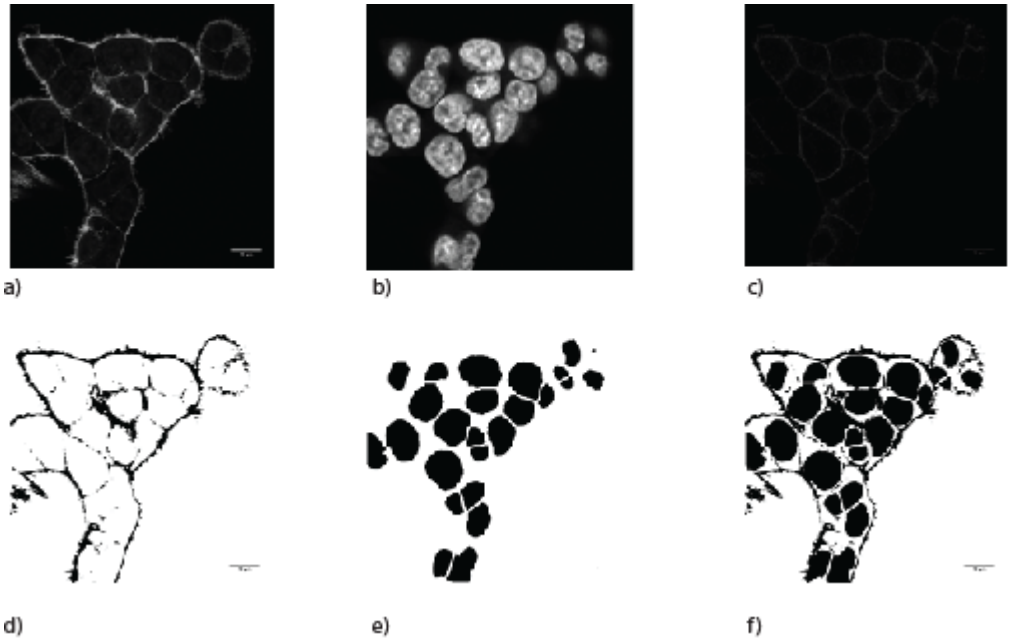



*Figure A1.1: Shows visual identification of the membrane compartment using Alexa-fluor 568 phalloidin in a) and identification of the nuclear compartment using DAPI in b) in HCT116 (wild-type) cells. Figure 1c) shows β-catenin stain in HCT116 (wild-type) cells. The corresponding threshold "masks" can be seen below to represent the membrane compartment in 1d), the nuclear compartment in 1e) and the cytoplasmic compartment in 1g).*

Once markers of the cellular compartments were identified, I aimed to assess whether their use could produce reliable and consistent measurement of my target proteins between different samples. To quantify the intracellular distribution of my target proteins, the quantity at the membrane region was assigned a value 1. Protein levels in the cytoplasmic and nuclear regions were then calculated relative to levels at the membrane.

This method of image analysis produced inconsistent quantification of relative intracellular distribution of proteins of interest. Figure A2 shows the distribution of β-catenin in untreated HCT116-HaB92 (wild-type) cells taken from five separate slides.

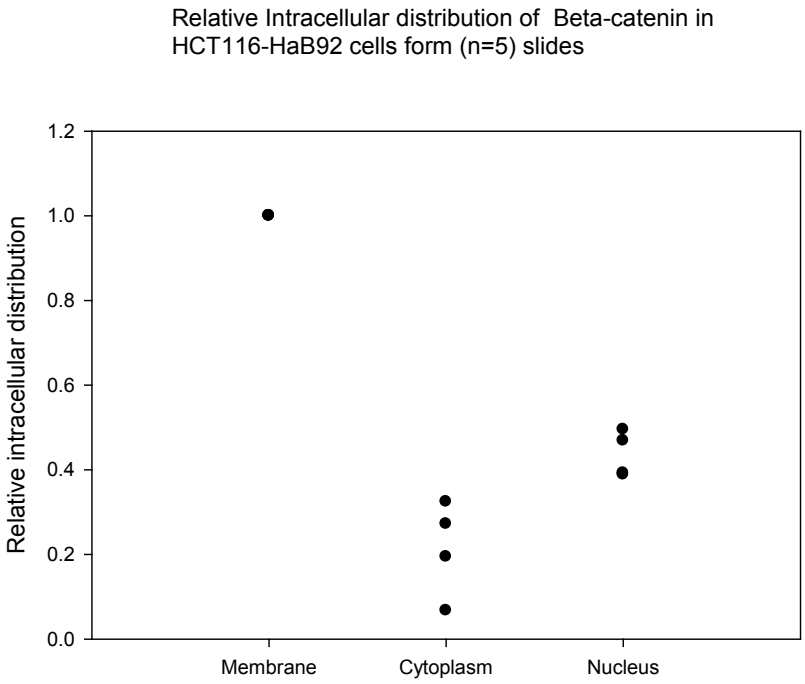



*Figure A1.2: Shows relative intracellular distribution of β-catenin in HCT116-(wild-type) cells taken from five separate slides. The membrane value is set at 1 and the cytoplasmic and nuclear content are measured relative to the membrane.*

Several problems were encountered using this method. Accuracy could be improved by selecting a single image slice for quantification. This, however, was not representative of the whole cell. Analysis of the entire stack was very time consuming if the process was carried out manually. In conjunction with image analysis experts, I developed a macro that was capable of automating the analysis process on the whole stack. Although this sped up the process, results were variable and unreliable.

The method was capable of detecting large changes in the localisation of β-catenin. For example I treated the cells with Leptomycin B, which acts to trap β-catenin in the cell nucleus. This large shift was detectable using the image analysis method described. Subtle changes produced by biochemical depletion of proteins of interest were not amenable to accurate measurement. I used siRNA to deplete APC. To determine successful transfection of the cell I used fluorescently tagged siRNA to provide a visual indication of transfection. The transfection pattern was

heterogeneous, so quantification of a whole field of cells was unrepresentative of treatment effects. Automated identification and quantification of successfully transfected cells was not possible. This added to inaccuracies inherent in the method.

**Quantification of β-catenin distribution in mouse intestine**

I also attempted to quantify the distribution of β-catenin in mouse gut tissue. I had aimed to be able to detect and quantify differences in distribution between normal gut tissue and that from APC $^{(min/+)}$ mice. Unfortunately, this was also fraught with problems. I tried to make adaptations to overcome these, but was ultimately unsuccessful. I will outline some of the issues and attempted solutions here.

**Automated identification of cell compartments**

I used actin and DAPI as markers of the cell membrane and nucleus, respectively. I then used image analysis programmes to apply a mask automatically to each of these markers based on signal intensity from the actin and the DAPI. This mask was then used as a template to represent each cellular region of interest. I aimed to quantify the β-catenin intensity contained within each region of interest to calculate its relative cellular distribution. The intention of using an automated approach was to minimise the inherent bias in delineating cell compartments manually. Several problems were encountered.

The cells in gut tissue are very closely and irregularly packed. Often a region of one cell (e.g. membrane) overlaps with nuclear or cytoplasmic regions of neighbouring cells. It was therefore impossible accurately to resolve each cellular region, rendering the produced data unreliable. A representative image of a mouse gut crypt can be seen in Figure A3.

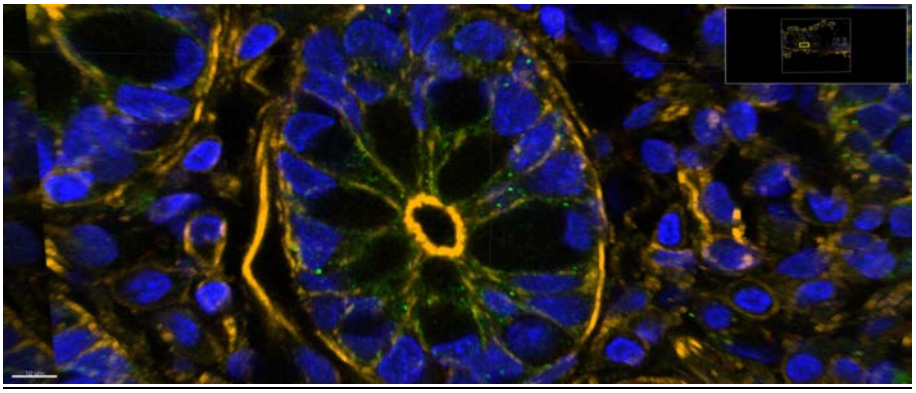


*Figure A1.3: Colonic crypt from normal mouse gut tissue stained with actin (yellow), DAPI (blue) and β-catenin (green) shows how closely overlapping cells and their components are.*

I attempted using both wax and frozen tissue sections, but this did not improve compartment delineation to an extent that made automatic determination of cell compartments feasible. I used multiple image analysis software packages including imageJ and Imaris. The new Imaris package was purchased to aid automatic analysis. I discussed the issues with all available image analysis experts within the College of Life Sciences, including the microscopy department and the OMERO team. I also arranged a discussion of possible data analysis solutions with an image analysis expert from the Imaris Company. None of these approaches yielded any success for tissue analysis.

**Manual Identification of Cell Compartments**

I then attempted manual identification of cell compartments, again using actin and DAPI as markers as an automated image analysis method was not possible. This involved drawing around the relevant marker and using the image analysis software to calculate β-catenin intensity contained within my manually acquired region of interest. A representative image of manually compartmentalised regions of interest is shown in Figure A4.

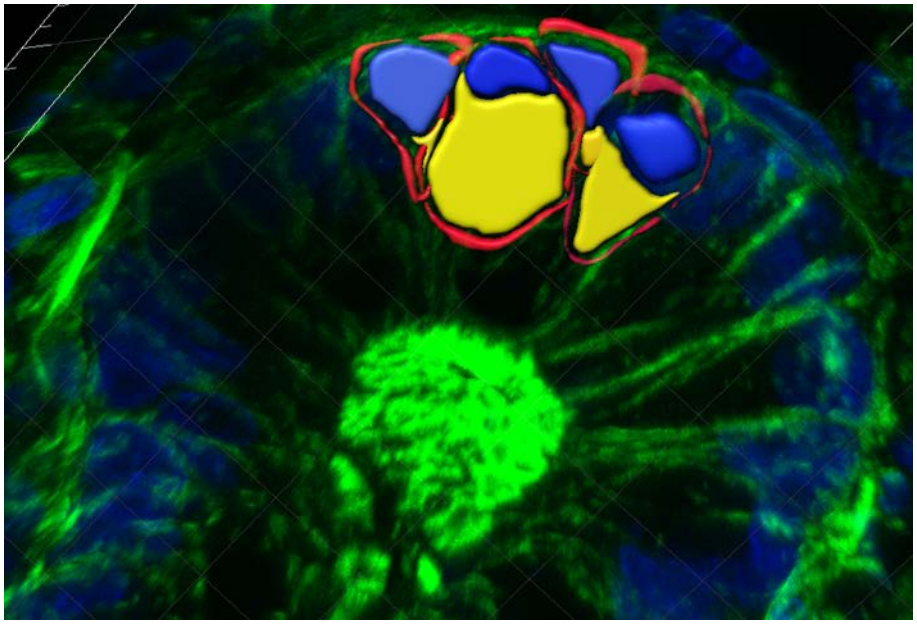


*Figure A1. 4: Identification of cell regions using manual compartmentalisation. Two Paneth cells and two stem cells are shown divided into regions representing nucleus (blue), membrane (red) and cytoplasm (yellow) from a colonic crypt in normal mouse gut tissue.*

Several problems occurred using this approach also. Frequently, cell regions overlap with other regions in neighbouring cells. It is thus very difficult accurately to identify a region of interest, even manually and when using whole stacks or individual *Z* planes. Even in a single *Z* plane it is challenging to unambiguously identify all regions reliably, making data unreliable, and without three-dimensional data the results do not represent the cell as a whole.

In tissue sections, in the smaller stem cells the cell volume is almost entirely filled with the nucleus. It is therefore difficult to identify a cytoplasmic compartment and identify it as a region of interest. Cellular identification was based on gross morphological features. Paneth cells are characterised by large empty-looking cytoplasmic regions and stem cells are the small intervening cells. The alternative is to use specific cell-type markers such as lysozyme and LGR5. However, these then occupy two fluorescent channels, limiting the availability of other fluorescent microscope channels required for actin, DAPI and proteins of interest such as β-

catenin. As the analysis was making use of tissue sections, it is impossible to determine with confidence what position in the crypt is being scrutinised. An estimate of this can be made using the crypt lumen diameter, but this is open to error. Having an accurate estimate of this is important as crypt position could influence the cellular distribution of β-catenin. Another consideration in this image analysis was time. To analyse a single crypt in this manner took a considerable amount of time. Given the unreliable nature of the data produced and the number of crypt measurements that would be required, I abandoned this approach. Not surprisingly, analysing the data acquired in this manner showed large inter-crypt variability, consistent with the issues raised above, and questioning the robustness of the data (Figure A5).

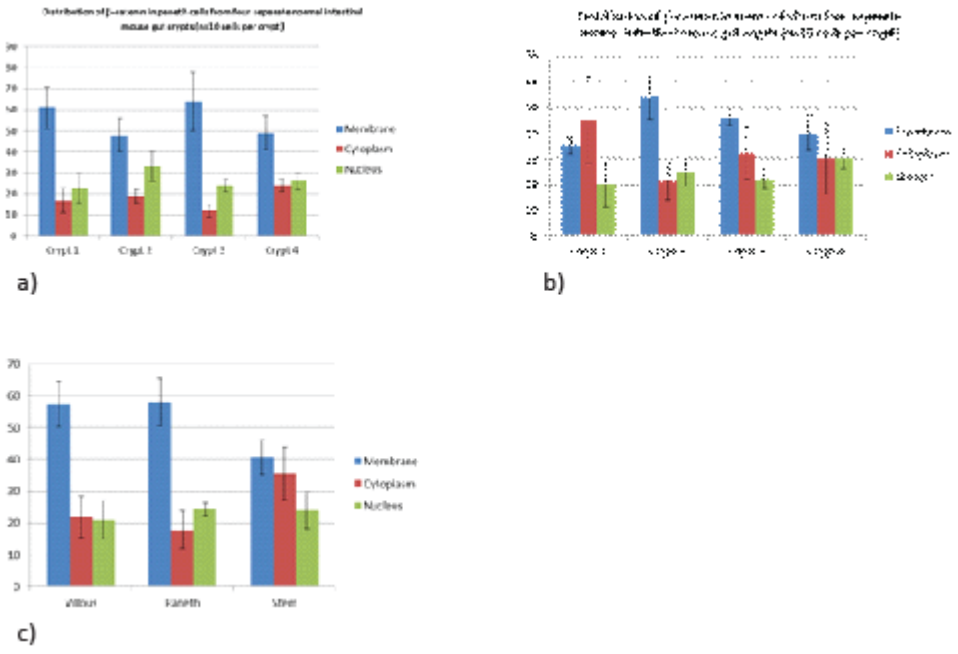


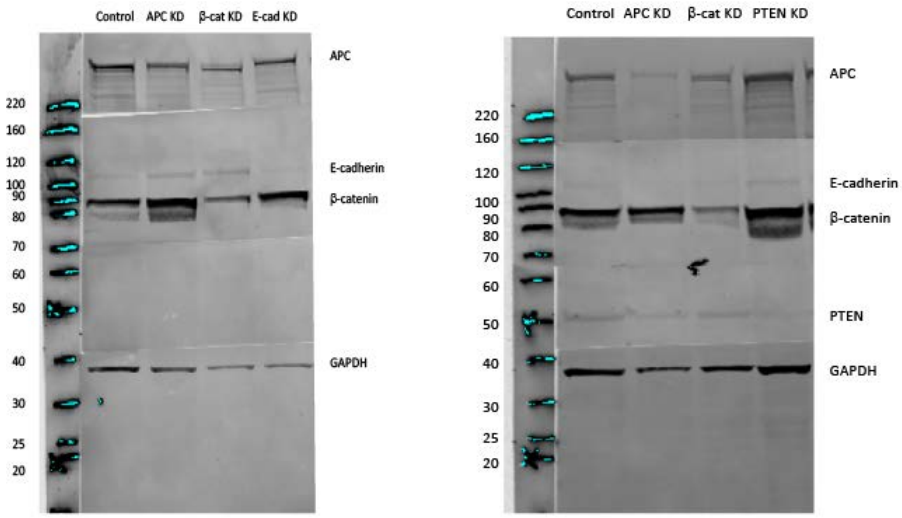

*Figure A1.5: β-Catenin distribution in cells from normal intestinal mouse crypts. Comparison between Paneth cells from four different crypts (n = 10 cells per crypt) is shown in a) and between stem cells in b). Figure A5c) shows β-catenin distribution between cells from the villous, Paneth cells and stem cells (n = 30 cells from each region).*

Further advances are required for accurate quantification of β-catenin using immunofluorescence imaging. Although β-catenin has a predominant membrane location, it has a diffuse distribution in other cell compartments also. Quantification of protein localisation using imaging may be more robust if the protein of interest had a more specific location. Good visual

representations of the distribution can be easily achieved, but accurate numerical quantification of this is problematic. Given the failure to develop a robust reliable method and the large time investment involved, it was decided that the project should be approached from a slightly different angle.

## Appendix 2

Throughout this thesis I have shown a number of western blots. These have generally been shown without an accompanying sizing ladder. Here I will show a few examples of full blots including ladders. The blue colour visible on the blots is due to pen used to mark the ladders. The first example shows efficiency of target protein knockdown following siRNA treatment (Figure A2. 1)




a) B)

*Figure A2.1: Western blot showing depletion of APC, β-catenin and E-cadherin compared to control in (a) and APC, β-catenin and PTEN in (b). Blots were probed with antibody targeting APC, E-cadherin, β-catenin, PTEN and GAPDH.*

Generally a knockdown of approximately 80-90% of control levels were achieved in each targeted protein. This was consistent across all cell lines studied. I also over expressed β-catenin in the mutant and wild-type cell lines. A whole representative blot including ladder is shown (Figure A2. 2)

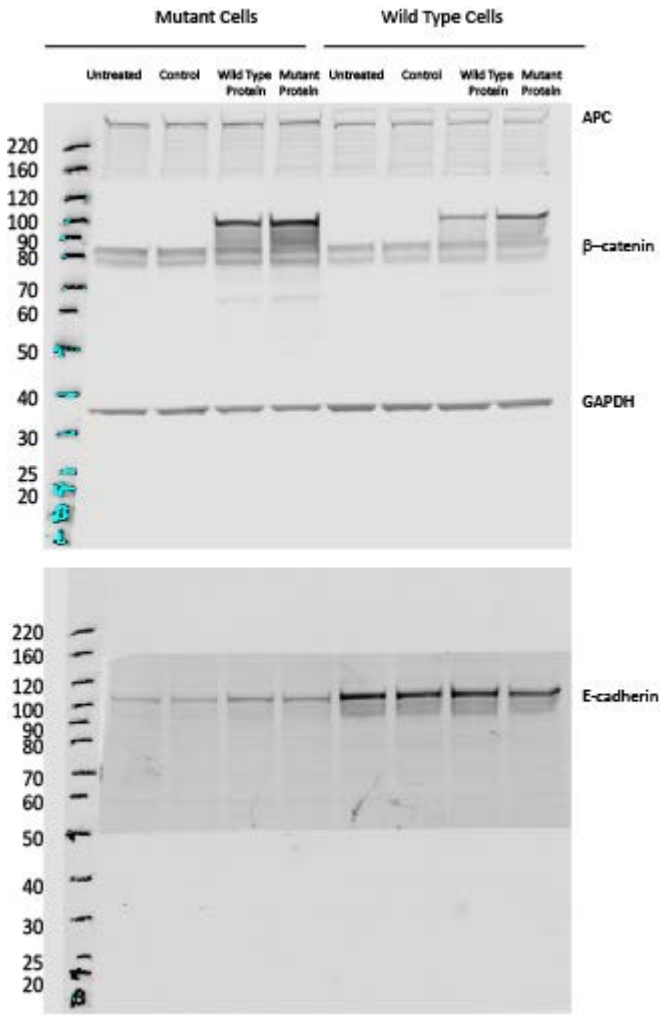



*Figure A2.2: Western blot showing expression of exogenous mutant or wild-type β-catenin in mutant (Left) and wild type (right cells). Blots were probed with antibodies against APC, β-catenin and GAPDH (Top panel). The same blot was also probed against E-cadherin (bottom panel).*

I also expressed exogenous PTEN protein in PTEN (-/-) cells. A full size representative western blot is shown (Figure A2. 3).

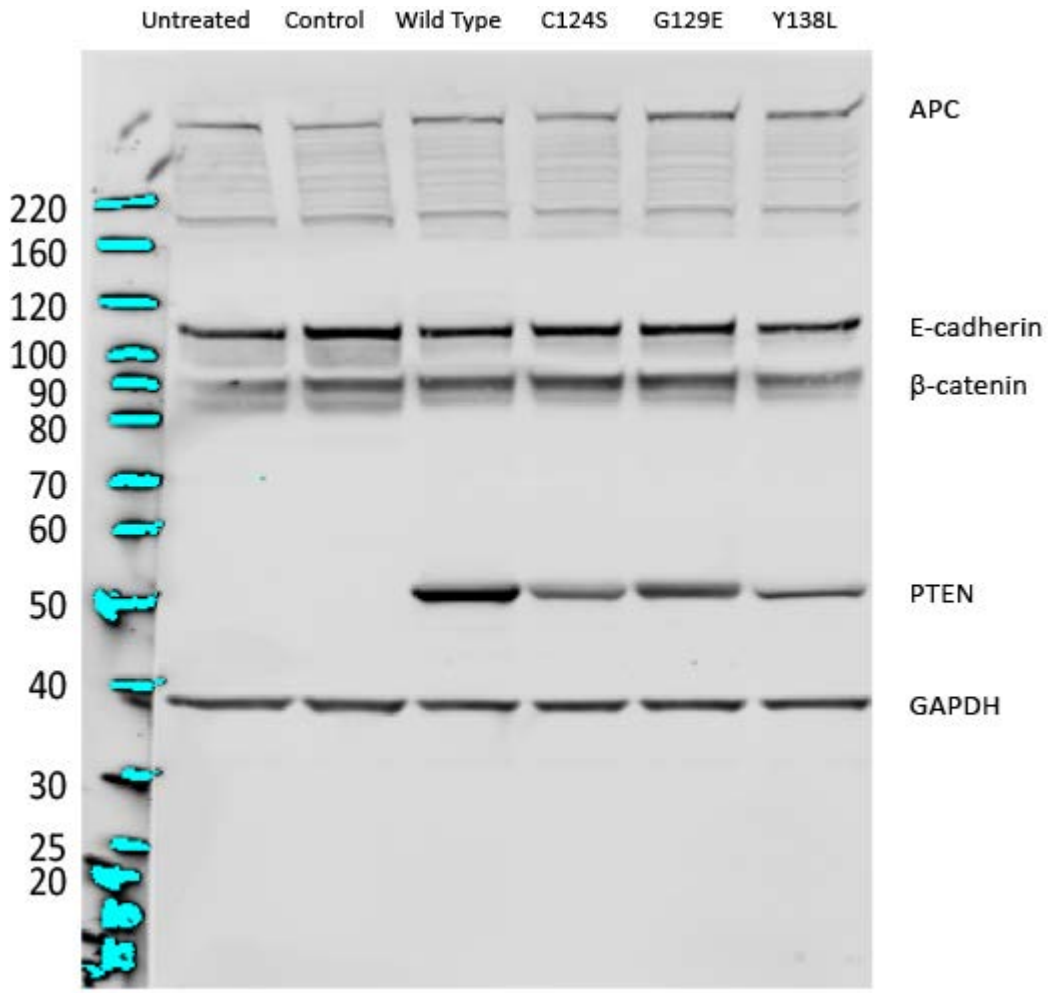



*Figure A2.3: Western blot showing expression of a series of PTEN proteins in PTEN (-/-) cells compared to untreated and control treated cells. Blots were probed with antibodies against APC, E-cadherin, β-catenin PTEN and GAPDH.*

I also fractionated cells into cytoplasmic, membrane, nuclear and cytoskeletal compartments. In order to assess the efficiency of the fractionation process I blotted against specific compartmental markers. I used GAPDH, E-cadherin, TOPO II and Keratin 19 as markers of the cytoplasmic, membrane, nuclear and cytoskeletal compartments respectively. After determining that the fractionation protocol was robust using these markers I did not blot for these four markers on every single blot. If I found an unexpected result I would check the fractionation efficiency with compartment markers. I also frequently blotted for E-cadherin, TOPO II and GAPDH to mark 3 of the 4 compartments. A representative blot is shown (Figure A2. 4).

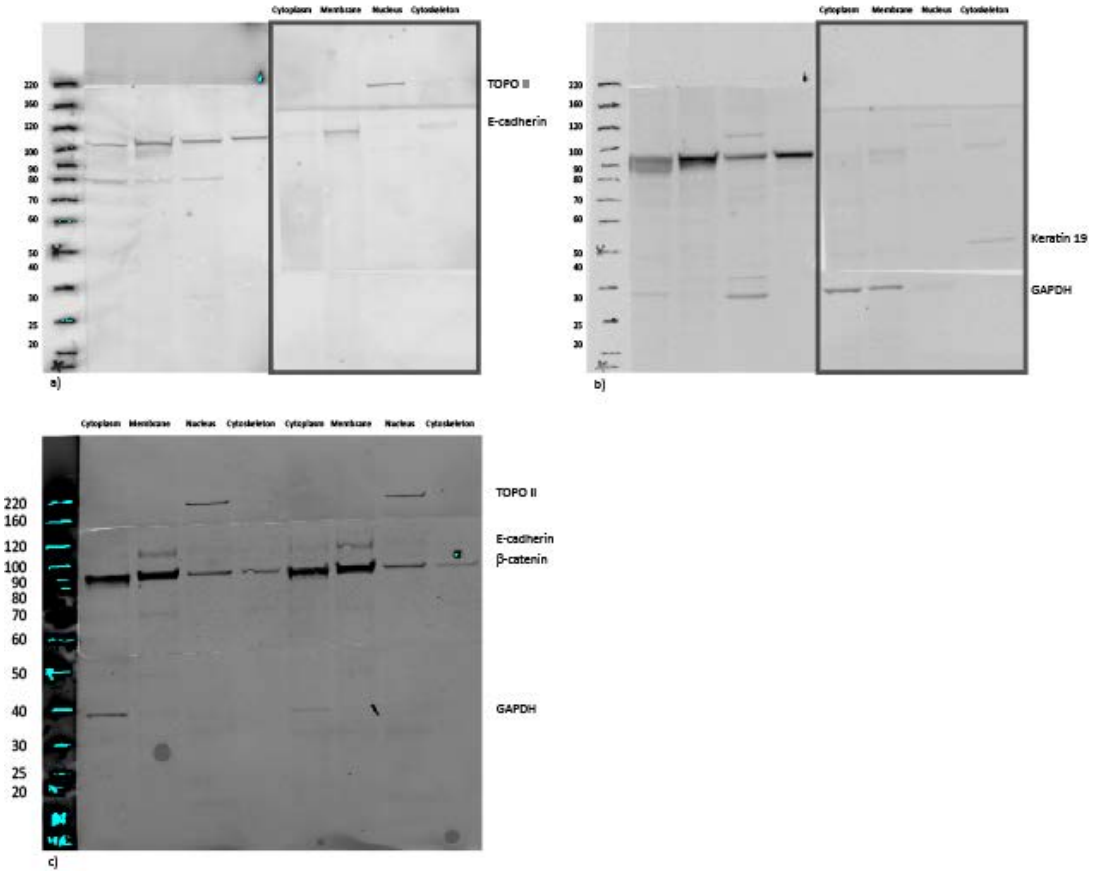



*Figure A2.4: Representative western blots showing compartmental markers. In (a) the membrane compartment is shown by blot against E-cadherin and the nuclear compartment by TOPO II. In (b) GAPDH shows the cytosolic compartment and Keratin 19 the cytoskeletal. In blot (c) 2 cell fractionations are shown. The blot is probed against ®-catenin and efficiency of fractionation is shown by blot against GAPDH, E-cadherin and TOPO II to show the cytosolic, membrane and nuclear compartments respectively.*